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Molecular analysis of RAX2-regulated retinal development using human retinal organoids at a single-cell resolution

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Human embryonic stem cells (hESC)-derived retinal organoids are sophisticated *in vitro* systems for dissecting the complex dynamics of human retinal development. The formation of the human retina is a precisely organized process that depends on the regulated differentiation of retinal progenitor cells; however, many of the basic mechanisms remain to be explored. Here, using hESC-derived retinal organoids, we elucidated the temporal contribution of RAX2 to retinal development, with an emphasis on photoreceptor cells (PC) formation. The results were corroborated using human fetal retinal tissue at various gestational ages. Using CRISPR/Cas9-mediated gene knockout, we delineated the essential role of RAX2 in modulating PC specifications. RAX2 deficiency significantly altered the expression of PAX6 and SOX2, two essential regulators of retinogenesis. Our results suggested that RAX2 is significant in retinal development, underpinning its potential as a therapeutic target in related retinal disorders.

KEYWORDS

human embryonic stem cells (hESC), retinal organoid, retinal development, photoreceptor cells, ScRNA-seq

1 Introduction

The human retina, acting as a processor for integrating visual signals, orchestrates interactions among various retinal cell types in a delicate cellular structure. Retinogenesis is the process by which multipotent retinal progenitor cells (RPC) differentiate into specialized cells, including retinal ganglion cells (RGC), photoreceptor cells (PC, including rods and cones), Müller cells (MC), amacrine cells and bipolar cells. This process is meticulously orchestrated by a network of signaling pathways, as delineated in previous studies (Bassett and Wallace, 2012). Recent studies using bulk transcriptomic profiling, single-cell RNA sequencing (scRNA-seq), and single-cell assay for transposase-accessible chromatin sequencing (scATAC-seq) have systematically

examined the cellular composition and molecular expression patterns of the human retina and retinal organoids (RO) derived from human embryonic stem cells (hESC) (Voigt et al., 2021; Li et al., 2023; Wahle et al., 2023; Zhang et al., 2024). These investigations have provided critical insights into the spatiotemporal dynamics of cellular diversification, thereby offering an integrative framework for understanding the molecular mechanisms underlying retinogenesis and retinal disease pathogenesis. In a previous study, using scRNA-seq to analyze hESC derived RO at five different time points (day36-day186, D36-D186), we identified 9 cell populations, including RPC, RGC, PC, MCs, and retinal pigment epithelial (RPE) cell populations, and described the emergence, maturation, and regulation of RPC and PC populations in detail (Wang et al., 2021).

The retinal and anterior neural fold homeobox (RAX) gene family encodes homeodomain transcription factors, and is crucial for vertebrate retinal development. Through evolutionary analysis, jawed vertebrate RAX genes were classified into two distinct subgroups: RAX1 (commonly referred to as RAX) and RAX2 (Kon and Furukawa, 2020). RAX is initially expressed in the anterior neural fold and later in the embryonic diencephalon, which gives rise to the retina and pineal gland (Mathers et al., 1997). RAX is critical for retinal cell fate determination and the maturation and survival of PC (Irie et al., 2015). RAX-deficient mice exhibit severe forebrain malformations and lack optic vesicles (Mathers et al., 1997). Mutations in human RAX have been linked to congenital ocular disorders, including anophthalmia and microphthalmia (Voronina et al., 2004). RAX2 (also known as QRX) is required for retinal neurogenesis in *Xenopus* (Wu et al., 2009) and chicks (Sanchez-Arrones et al., 2009). Studies have found that RAX2 orthologs are essential for maintaining adult medaka fish retinal stem cells (Reinhardt et al., 2015). RAX2 protein physically interacts with the CRX protein synergistically to modulate the expression of PC-specific genes, such as *Rhodopsin*. Emerging clinical evidence has linked RAX2 mutations to various inherited retinal diseases (IRD). Dominant mutations, such as c.260G>A (p.Arg87Gln), have been associated with age-related macular degeneration (AMD), while variants like c.409G>C (p.Gly137Arg) and c.417_422dup (p.Pro140_Gly141dup) have been linked to cone-rod dystrophy (CRD) (Wang et al., 2004). The heterozygous c.465_475del (p.Ala156Argfs*131) variant, identified in familial cases of cone dystrophy or CRD, disrupts the N-terminal coding region of RAX2, potentially impairing its function as a CRX cofactor (Yang et al., 2015). Van de Sompele *et al.* demonstrated that biallelic RAX2 mutations, including c.155C>G (p.Pro52Arg), c.335dup (p.Ala113Glyfs*178), c.145 T>C (p.Ser49Pro), and g.3771337_3774298del, cause autosomal recessive retinitis pigmentosa (ARRP) (Van de Sompele et al., 2019). These mutations may impair the RAX2 protein folding, stability, and transactivation capability. Notably, RAX2 mutations are not compensated by RAX activity in human disease. Unlike humans, mice lack RAX2 orthologue, complicating functional studies (Wang et al., 2004). ScRNA-seq analysis of the human fetal neural retina revealed that RAX2 was primarily expressed in PC (Hu et al., 2019), which aroused our interest in exploring its potential role in human retinogenesis.

2 Materials and methods

2.1 Patients and tissue samples

The five human retinal specimens used in this study were obtained from voluntarily donated aborted fetuses, sourced from the Senior Department of Ophthalmology at the Third Medical Center of the Chinese PLA General Hospital. The Ethics Committee of the Third Medical Center of the Chinese PLA General Hospital approved this study (ID: KY 2021-021), and written informed consent was obtained from all participants. The procedures in this study adhered to the Helsinki Declaration of 1964 and its amendments, ensuring ethical integrity (World Medical, 2013).

2.2 hESC culture and RO differentiation

The hESC line H9 were routinely cultured in Essential 8 medium (ThermoFisher, A1517001) on plates coated with Vitronectin (Gibco, A14700). For passaging, cells were treated with Accutase (Stemcell Tech, 07920). RO differentiation followed established protocols with minor modifications (Wang et al., 2021; Kuwahara et al., 2015). Aggregates were cultured under 40% O₂/5% CO₂ conditions (30 aggregates per 10-cm dish) from day 24 (D24), using an NR-differentiation medium comprising DMEM/F12 (Gibco, 10565018), KSR (Gibco, 10828028), N2 supplement (Gibco, A1370701), 0.1 mM taurine (Sigma, T0625), and 0.5 μM retinoic acid (Sigma, R2625). Under these conditions, RO continued to grow for several weeks.

2.3 Establishment of genetically engineered hESC

Single guide RNAs (sgRNA) constructs targeting critical RAX2 were cloned into px459 plasmids (Addgene, 62988) for knockout cell generation. HESC were transfected with these sgRNA plasmids using the Lipofectamine Stem Transfection Reagent (Invitrogen, STEM00001) and exposed to 0.5 μg/mL puromycin for 48 h. 2,000–3,000 surviving cells were plated on a 6 cm dish, and 96 single colonies were picked up to a 96-well plate. Genomic DNA was extracted for PCR using specific primers:

Fw: CTTAGGGCGTGAGAAGGGAT;

Rv: CCCCACGCCCAATTAACAGA.

The PCR products were validated by TA cloning and Sanger sequencing to confirm RAX2 gene deletions.

3 Results

3.1 Highly-expressed RAX2 in PC within RO and human fetal retinal tissue

Our earlier investigation used an *in vitro* self-organization model of human RO derived from hESC, which mimicked human retinal development, to conduct an scRNA-seq analysis at five different time points during RO differentiation (D36, D66, D96, D126, and D186) (Wang et al., 2021). In this study, to delineate

the role of the RAX2 in retinogenesis, we reanalyzed the scRNA-seq data. Canonical markers were used to distinguish 6 cell clusters: RPC, Proliferating-RPC, PC, RGC, MCs and RPE cells (Supplementary Figures S1A, S1B). RAX2 was primarily detected in the PC population (Figure 1A). A gradual increase in RAX2 expression correlating with PC emergence in RO was observed (Figures 1B–D), consistent with the immunofluorescence (IF) staining of human RO, which also revealed a progressive increase in RAX2-positive cells (Figure 1E; Supplementary Figure S2A). Moreover, RAX2 expression patterns aligned with canonical PC markers, including *CRX*, *NR2E3* and *NRL* (Figures 1A,B). *CRX*-positive cells appeared at D36 in a human RO culture and gradually increased over time. The expression of *NRL* and *NR2E3* significantly increased during the maturation of PC (Wang et al., 2021), and *OTX2* was found to be involved in embryonic PC fate determination (Muranishi et al., 2011). To enhance our comprehension of RAX2 dynamics in retinal development, we obtained human retinal tissue from voluntarily donated aborted fetuses aged 12–24 weeks of gestation, and performed multi-immunofluorescence (multi-IF) staining to precisely track the temporal expression patterns of RAX2 (Figure 1F; Supplementary Figure S2B). A notable increase in RAX2-positive cells was observed from 20 to 24 weeks, coinciding with the reported initiation of PC development (Hu et al., 2019). These findings indicated that RAX2 may regulate PC maturation during retinal development.

3.2 Establishment of RAX2-knockout hESC utilizing CRISPR/Cas9-mediated gene editing

To explore the influence of RAX2 on human retinal development, the CRISPR/Cas9 system was used to disrupt critical exons of RAX2 in hESC (H9 cell line). Seven sgRNAs were created to target different regions around the gene, and their effectiveness was evaluated using a surveyor assay (Supplementary Figure S3). Cas9/sgRNA-7 and Cas9/sgRNA-6, both of which exhibited notable cleavage efficiencies, were selected for subsequent gene editing (Figure 2A). Two homozygous mutants, RAX2^{-/-}-1 and RAX2^{-/-}-2, were successfully generated and validated through Sanger sequencing (Figure 2B). Evaluation of genomic copy number variation (CNV) (Supplementary Figure S4), ESC colony morphology (Figure 2C), pluripotency markers expression (Figure 2D), and cell proliferation (Figure 2E) showed no significant differences between RAX2^{-/-} and wild type (WT) hESC. Embryoid body (EB) formation assay (Figures 2F,G) revealed that RAX2 deficiency in hESC significantly reduced the expression of ectoderm markers in the derived EBs, including *MAP2*, *PAX6*, *RAX*, and *SIX6* (Figure 2H). This finding underscored the essential function of RAX2 in the ectoderm-related differentiation process.

3.3 RAX2 deficiency affects PC fate determination during RO differentiation

Using a previously established BMP4-induced RO self-organization protocol (Wang et al., 2021), WT and RAX2^{-/-} hESC

were grown in a 3D culture for 66 days (Figure 3A), and twenty-four RO were harvested for scRNA-seq analysis from each of WT and RAX2^{-/-} group. Following a rigorous quality control evaluation and removal of doublets, a UMAP analysis revealed five primary cell clusters, with the cell types identified through enriched gene profiles and canonical markers (Figures 3B,C). A marked reduction in RAX2 expression was detected in all the RAX2^{-/-} hESC-derived RO cell clusters identified (Figure 3D). A significant decrease in the percentage of PC, RPC, and RPE cell populations was observed in RO derived from RAX2^{-/-} hESC compared to the those from WT hESC (Figure 3E). Considering the process of retinal development, we focused on RPC, RGC, and PC clusters (Figures 3F,G). A developmental pseudotime trajectory analysis was conducted, which helped reveal highly interconnected nodes potentially indicating the differentiation status (Figures 3H,I). Depletion of RAX2 significantly altered various cellular distributions. Differentiation into PC was notably affected by the absence of RAX2, leading to a bias towards RGC lineage commitment. Additionally, the PC population analysis revealed a decrease in pathways associated with PC differentiation (Figure 3J). RT-qPCR analysis demonstrated decreased expression of PC-specific markers (*CRX*, *NRL*, and *NR2E3*) in RO derived from RAX2^{-/-} hESC, alongside elevated levels of RGC markers (*POU4F2* and *THY1*), consistent with the observed lineage bias (Supplementary Figure S5). These findings underscored the critical function of RAX2 in PC fate determination.

3.4 RAX2 regulates the expression of PAX6 and SOX2 during RO differentiation

In our previous study, we observed that the proportion of each cell type, including PC, in RAX2^{-/-} hESC-derived RO differed from that of the WT RO (Figure 3E), suggesting that RAX2 influenced the differentiation state of the entire organoid. To elucidate the underlying mechanism, we analyzed differentially expressed genes and noticed that the expression patterns of *PAX6* (Oron-Karni et al., 2008) and *SOX2* (Diacou et al., 2022), both vital for eye development, were significantly altered by RAX2 deficiency (Figure 4A). RT-qPCR and Western blot analyses confirmed the reduced expression of *PAX6* and *SOX2* (Figures 4B,C). In addition, IF staining analysis of human RO at D66 revealed a marked decrease in the fluorescence intensity of *PAX6* and *SOX2* in RAX2^{-/-} hESC-derived RO (Figure 4D). Overall, our results suggested that RAX2 is critical for retinal development by modulating *PAX6* and *SOX2* expression.

4 Discussion

In this work, we systematically examined the expression patterns of RAX2 in human fetal retinal tissue and hESC-derived RO at different stages. By integrating bioinformatics analyses with biochemical assays of RNA and protein levels in RAX2-deficient hESC-derived RO, we delineated RAX2 as a pivotal determinant of PC specification. Notably, the loss of RAX2 significantly altered the proportions of various cell populations within the RO. The scRNA-seq results, validated through RT-qPCR, Western blotting,

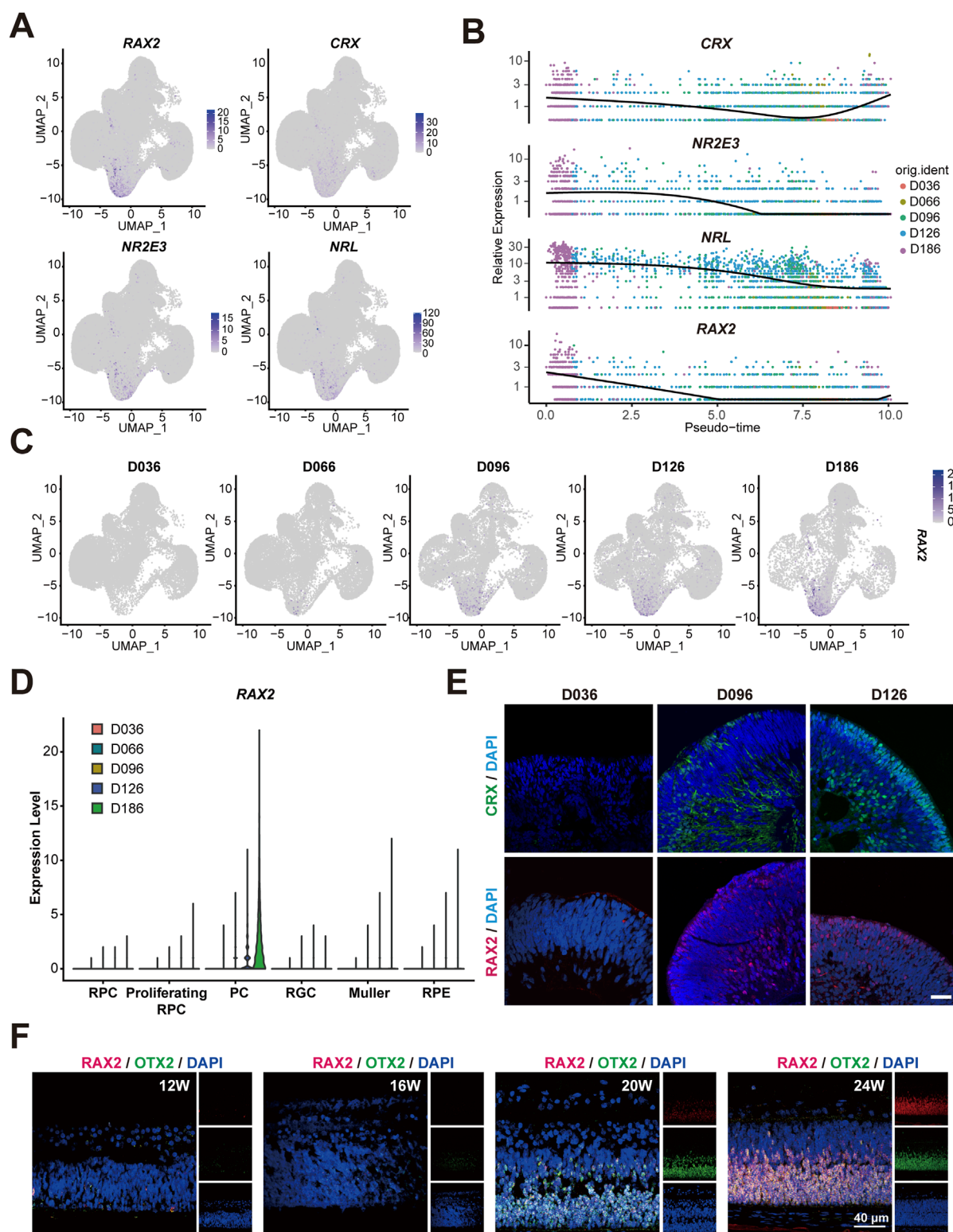


FIGURE 1

Highly expressed *RAX2* in PC during early stages of RO and tissue. (A) Multiple feature plots of RO display integrated expression profiles across five timepoints (D036, D066, D096, D126, D186), highlighting the expression of *RAX2* and the hallmark genes of the PC population, including *CRX*, *NRL* and *NR2E3*, in hESC-derived RO. (B) Pseudotemporal trajectory map exhibiting the expression of *RAX2* and marker genes of PC population, including *CRX*, *NRL*, and *NR2E3* in hESC-derived RO at different timepoints (D036, D066, D096, D126, D186). (C) Multiple feature plots exhibiting *RAX2* expression in hESC-derived RO at different timepoints (D036, D066, D096, D126, D186). (D) Violin plots exhibiting *RAX2* gene expression in hESC-derived RO at different timepoints (D036, D066, D096, D126, D186). (E) Representative IF-staining images of *CRX* and *RAX2* in hESC-derived RO at different timepoints (D036, D096, D126). Scale bars, 40 μ m. (F) Representative IF-staining images of *RAX2* and *OTX2* in human retinal tissue from aborted fetuses, spanning gestational ages of 12–24 weeks. Scale bars, 40 μ m. W: weeks.

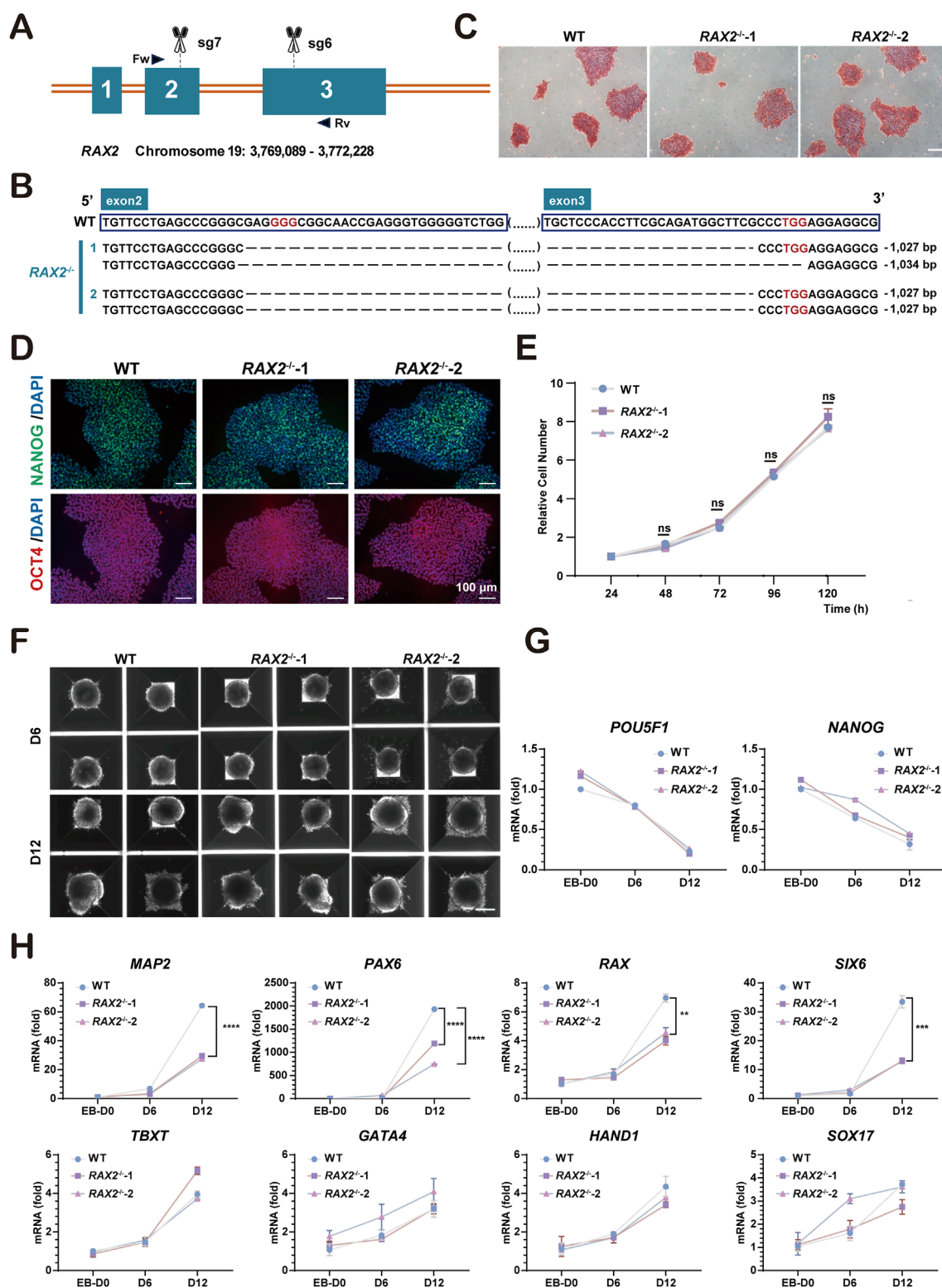


FIGURE 2

Establishment of *RAX2*-knockout hESC. (A) Schematic illustration of knocking out *RAX2* in hESC by CRISPR/Cas9 system. Scissors indicate the sgRNAs; boxes represent the exons; triangular arrows represent primers. Fw: forward primer; Rv: reverse primer. (B) Sanger sequencing results for *RAX2*^{-/-} and WT hESC clones. Red words indicate the PAM sequence; ellipses in parentheses indicate sequences that are not listed; dashed line indicates deleted bases. (C) Alkaline phosphatase staining of *RAX2*^{-/-} and WT hESC clones. Scale bars, 100 μ m. (D) Representative IF-staining images of pluripotency markers in *RAX2*^{-/-} and WT hESC clones. Scale bars, 100 μ m. (E) Cell proliferation rate of *RAX2*^{-/-} and WT hESC clones (n = 3 independent experiments). ns, not significantly different. (F) Representative images of EB formation assay for *RAX2*^{-/-} and WT hESC clones. Scale bar, 200 μ m. (G) RT-qPCR analysis for *POU5F1* and *NANOG* expression in *RAX2*^{-/-} and WT hESC-derived EBs at different timepoints (D0, D6, D12). (H) RT-qPCR analysis for ectoderm, endoderm, and mesoderm markers expression in *RAX2*^{-/-} and WT hESC-derived EBs at different timepoints (D0, D6, D12).

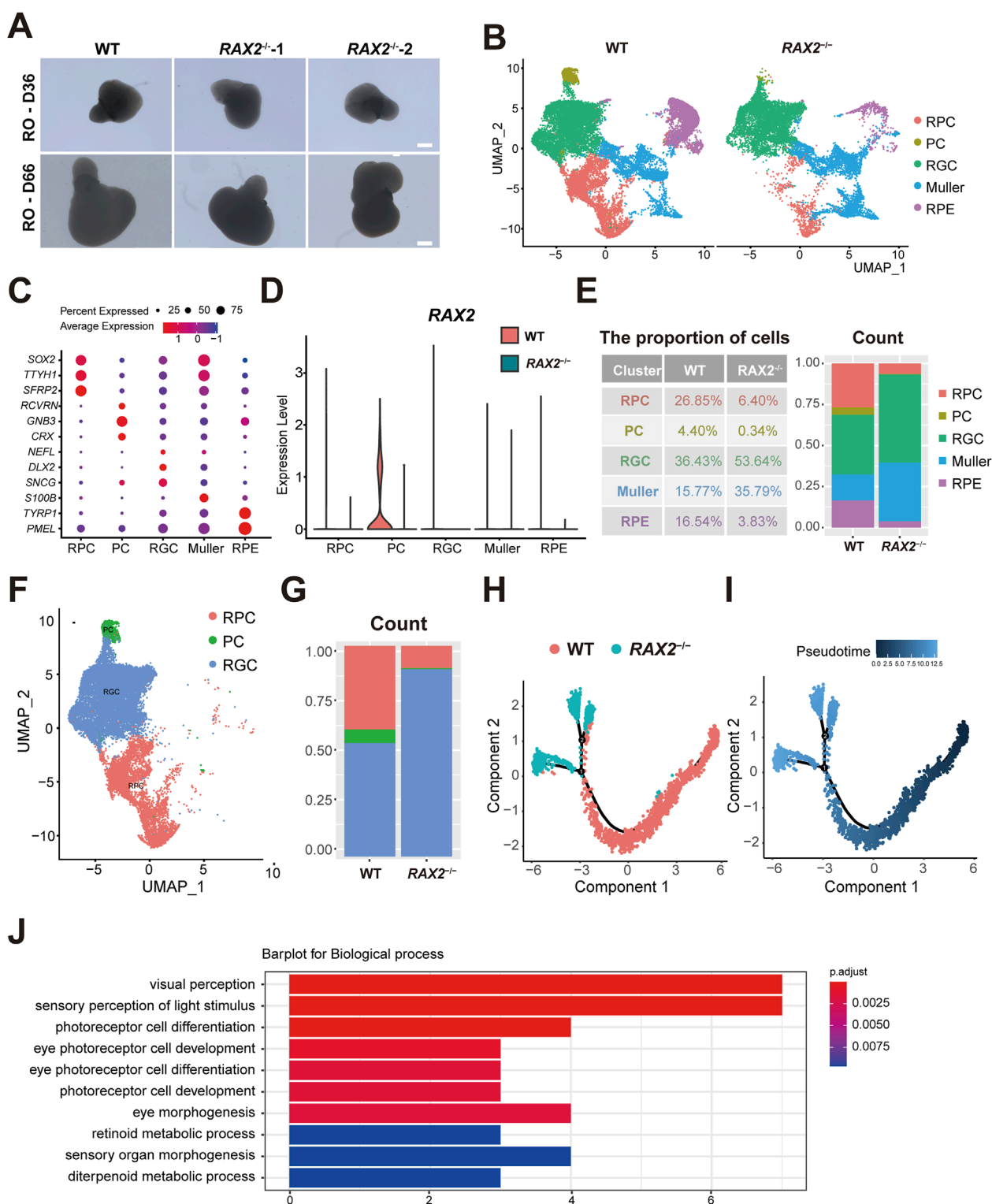


FIGURE 3

The absence of $RAX2$ affects PC fate determination. (A) Representative images of $RAX2^{-/-}$ and WT hESC-derived human RO at D36 and D66. Scale bars, 100 μ m. (B) UMAP plots of the $RAX2^{-/-}$ and WT hESC-derived RO in D66, labeled by cell types. (C) Dot plots for the marker genes expression by cell types. The color represents the average expression level; the size of dot represents the percentage of cells within a cell type. (D) Violin plots for $RAX2$ expression in clusters from $RAX2^{-/-}$ and WT hESC-derived RO. (E) Proportion of each cell types from $RAX2^{-/-}$ and WT hESC-derived RO in D66. (F) UMAP plot of the RPC, PC and RGC clusters from the $RAX2^{-/-}$ and WT hESC-derived RO in D66, labeled by cell types. (G) Percentages of the RPC, PC and RGC clusters from the $RAX2^{-/-}$ and WT hESC-derived RO in D66. (H) The Monocle 2 trajectory plot showing the pseudotemporal ordering of cluster RPC, RGC and PC from $RAX2^{-/-}$ and WT hESC-derived RO in D66. Numbers in black circles indicate the different cell status numbers. (I) Pseudotemporal ordering trajectory map of RPC, RGC and PC clusters from $RAX2^{-/-}$ and WT hESC-derived RO in D66. The colors from dark to light indicate the pseudotime order. (J) GO analysis of the top 10 downregulated biological processes in PC subset from the $RAX2^{-/-}$ and WT hESC-derived RO at D66. Horizontal axis values the count of enriched genes per term.

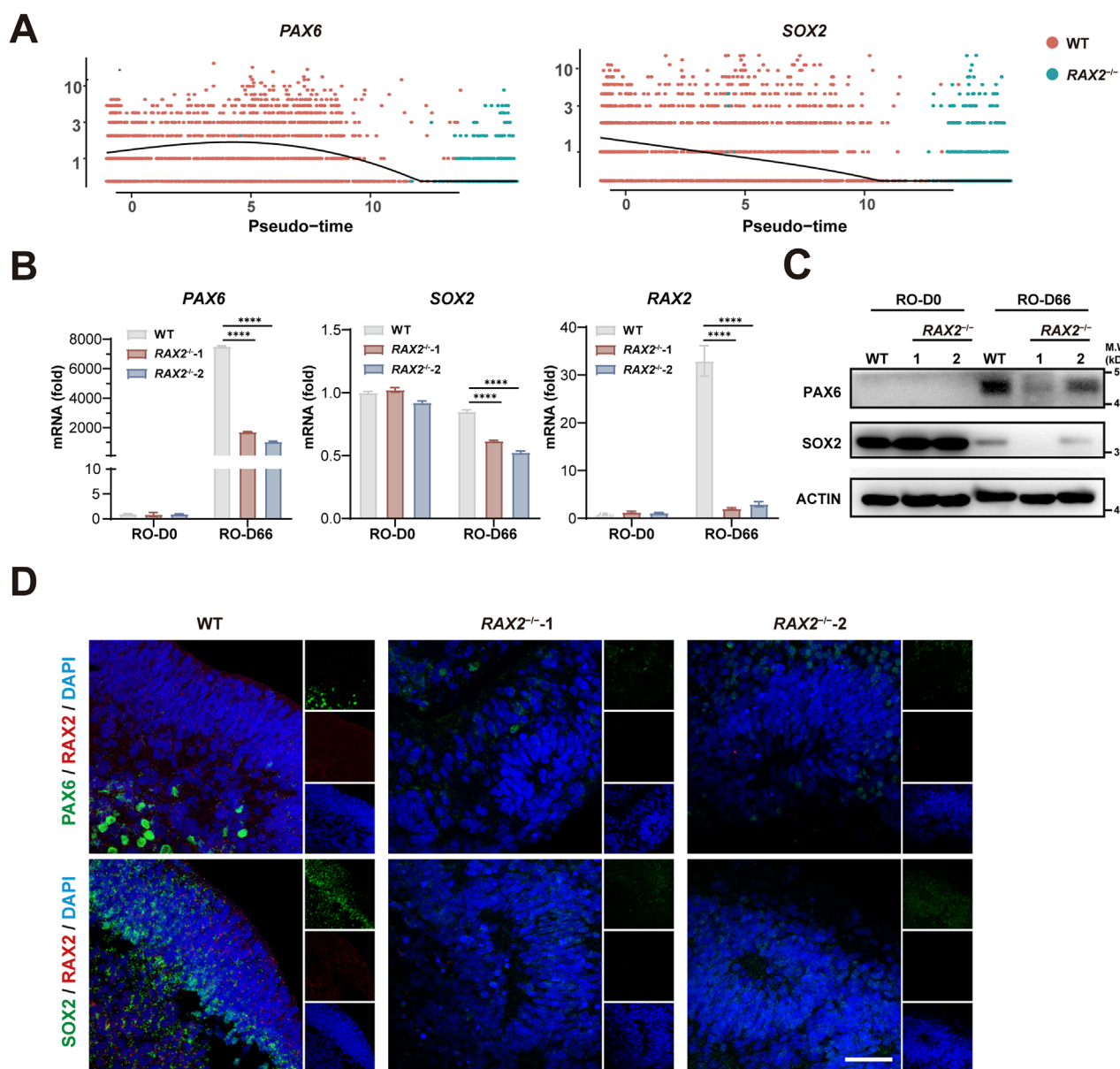


FIGURE 4

RAX2 regulates PAX6 and SOX2 expression. (A) Expression of SOX2 and PAX6 from $RAX2^{-/-}$ and WT hESC-derived RO over pseudotime. (B) RT-qPCR analysis for the expression of RAX2, PAX6, and SOX2 in RO derived from $RAX2^{-/-}$ and WT hESC at D66. All experiments were repeated in three batches of organoids. (C) Expression of PAX6, and SOX2 in RO derived from $RAX2^{-/-}$ and WT hESC at D66 was detected by Western blot. All experiments were repeated in three batches of organoids. (D) Representative images of IF staining of RAX2, PAX6, and SOX2 in RO derived from $RAX2^{-/-}$ and WT hESC at D66. Scale bars, 40 μ m. All experiments were repeated in three batches of organoids.

and IF staining, demonstrated that these alterations correlated with reduced expression of PAX6 and SOX2, which are key regulators in retinal development. The precise modulation of PAX6 and SOX2 expression within optic cup progenitors is essential for retina development, with a release of neural potential in the retina (Klimova and Kozmik, 2014; Oron-Karni et al., 2008). The spatial and temporal regulation of PAX6 expression, however, remains incompletely understood, suggesting that the regulatory function of RAX2 may be more complex than previously appreciated (Wang et al., 2004).

Our observations suggest that alterations in RAX2 expression are vital for retinal development, particularly in PC. Previous researches have shown the specific co-expression patterns of Rax2 and Vsx2 in defining retinal cell identity (Pandit et al., 2015), with external signals like BMP activity influencing RAX2 expression in chicks and zebrafish (Bielen and Houart, 2012). In the human retina, RAX2 is present in the outer and inner nuclear layers and serves as a PCE-1-binding protein, partnering with CRX and NRL to manage the expression of photoreceptor genes (Wang et al., 2004). Given the complex interplay among retinal cells and minor deviations may

disrupt homeostasis, the deletion of *RAX2* could create cascading effects on retinal cell viability, thus affecting the progression of retinal development.

Our findings have significant translational relevance due to their potential for supporting retinal diseases treatments involving photoreceptor loss, such as retinitis pigmentosa (Klymenko et al., 2024) and AMD (Tan et al., 2023). *RAX2* expression modulation may provide dual effects of both preventing photoreceptor degeneration and promoting their regeneration. Future studies should investigate the role of *RAX2* in ocular development, develop therapies by expressing the human *RAX2* gene in *Rax*-deficient mice, and generate disease models using RO. Understanding *RAX2*'s interactions with key developmental genes like *PAX6* and *SOX2* is crucial for advancing gene therapy approaches for retinal disorders.

Our study acknowledges limitations in fully delineating the molecular interactions of *RAX2*. Future research using advanced genetic techniques and precise temporal analysis will be essential for elucidating the detailed mechanisms underlying this genetic pathway in retinal development. This study highlights the importance of further exploring the regulatory functions and interactions of *RAX2* to improve our comprehension of retinal development and discover new therapeutic interventions for retinal disorders linked to these cells.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the Third Medical Center of the Chinese PLA General Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SW: Investigation, Conceptualization, Data curation, Funding acquisition, Writing – original draft. YS: Investigation, Writing – original draft, Data curation. JN: Methodology, Resources, Writing – original draft. YH: Writing – review and editing, Project administration, Funding acquisition. GL: Project administration, Resources, Conceptualization, Funding acquisition, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2025.1609826/full#supplementary-material>

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