• Basic Research •

Cone-rod homeobox transcriptionally activates TCF7 to promote the proliferation of retinal pigment epithelial and retinoblastoma cells *in vitro*

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Abstract

● **AIM:** To investigate the proliferation regulatory effect of cone-rod homeobox (CRX) in retinal pigment epithelium (RPE) and retinoblastoma (RB) cells to explore the potential application and side effect (oncogenic potential) of CRX-based gene therapy in RPE-based retinopathies.

● **METHODS:** Adult human retinal pigment epithelial (ARPE)-19 and human retinal pigment epithelial (RPE)-1 cells and Y79 RB cell were used in the study. Genetic manipulation was performed by lentivirus-based technology. The cell proliferation was determined by a CellTiter-Glo Reagent. The mRNA and protein levels were determined by quantitative real-time polymerase chain reaction (qPCR) and Western blot assay. The transcriptional activity of the promoter was determined by luciferase reporter gene assay. The bindings between CRX and transcription factor 7 (TCF7) promoter as well as TCF7 and the promoters of TCF7 target genes were examined by chromatin immunoprecipitation (ChIP) assay. The transcription of the TCF7 was determined by a modified nuclear run-on assay.

● **RESULTS:** CRX overexpression and knockdown significantly increased (n=3, P<0.05 in all the cells) and decreased (n=3, P<0.01 in all the cells) the proliferation of RPE and RB cells. CRX overexpression and knockdown significantly increased and deceased the mRNA levels of Wnt signaling target genes [including MYC proto-oncogene (*MYC*), *JUN*, FOS like 1 (*FOSL1*), *CCND1*, cyclin D2 (*CCND2*), cyclin D3 (*CCND3*), cellular communication network factor 4 (*CCN4*), peroxisome proliferator activated receptor delta (*PPARD*), and matrix metallopeptidase 7 (*MMP7*)] and the luciferase activity driven by the Wnt signaling transcription factor (TCF7). TCF7 overexpression and knockdown significantly increased and decreased the proliferation of RPE and RB cells and depletion of TCF7 significantly abolished the stimulatory effect of CRX on the proliferation of RPE and RB cells. CRX overexpression and knockdown significantly increased and decreased the mRNA level of TCF7 and the promoter of TCF7 was significantly immunoprecipitated by CRX antibody.

● **CONCLUSION:** CRX transcriptionally activates TCF7 to promote the proliferation of RPE and RB cells *in vitro*. CRX is a potential target for RPE-based regenerative medicine. The

potential risk of this strategy, tumorigenic potential, should be considered.

● **KEYWORDS:** retinal pigment epithelial cell; retinoblastoma; cone-rod homeobox; transcription factor 7; regenerative medicine; tumorigenic potential

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INTRODUCTION

Retinal pigment epithelium (RPE) is the outermost layer of the retina that is composed of a single layer of RPE cells[1]. RPE play essential roles in maintaining normal functions of retina: supporting photoreceptors, regulating transport across the blood-retina barrier, and preventing internal nerves from ultraviolet light, for example[2-3]. Disorders involved in degeneration of RPE cells, including retinitis pigmentosa (RP), age-related macular degeneration (AMD), and Stargardt disease, are the leading causes of blindness worldwide[4-5]. So far, no significant therapeutic strategy has been developed for these degenerative diseases. Although it has been recognized that RPE cells are normally dormant, recent evidence indicates that quiescent RPE cells can reproliferate under a variety of circumstances, such as RPE atrophy, damage, *etc*[6-7]. Thus, gene and drug therapies keep the promise for these diseases and identification of the potential drug targets involved in PRE cell proliferation is urgently needed.

Cone-rod homeobox (*CRX*) gene encodes a photoreceptor-specific transcription factor which plays crucial roles in differentiation and survival of photoreceptors[8]. Loss-of-function mutations of *CRX* causes cone-rod dystrophy (CRD), another leading cause of blindness worldwide, as the result of concomitant loss of both cone and rod photoreceptors[9]; thus *CRX* has been recognized as a drug target for regenerative therapy of CRD. Recently, accumulating evidence supported the expression of *CRX* in RPE cells[10-12]. However, rare studies have been performed to explore the roles of *CRX* in RPE cells and whether *CRX* could be served as a potential drug target for regenerative therapy of RPE-related disorders has remained elusive.

The potential side effects of regenerative medicine, such as tumorigenic potential, have to be considered. Recent evidence supported that cone precursors were involved in the origin of retinoblastoma (RB)[13-14]. Considering the connection between RPE and photoreceptor cells, the side effect of oncogenic potential of RPE-based regenerative medicine has to be considered.

The objectives of this study are to explore whether *CRX* is a potential drug target for RPE-based regenerative medicine and whether these is oncogenic potential in *CRX*-based regenerative medicine for RPE-related degenerative disorders by investigation of the proliferation regulatory effects of *CRX* in both RPE and retinoblastoma cells.

MATERIALS AND METHODS

Cell Culture Human RPE cell lines [adult human retinal pigment epithelial (ARPE)-19 and human retinal pigment epithelial (RPE)-1] and human RB cell line, Y79, were obtained from American Type Culture Collection. The ARPE-19 and RPE-1 cells were verified by the expression of RPE-specific markers, retinaldehyde binding protein 1 (RLBP1) and RPE65. ARPE-19 and RPE-1 cells were cultured in a mixture of Dulbecco's modified Eagle's medium/F-12 (Thermo Fisher Scientific, USA) with GlutaMAX (Thermo Fisher Scientific). Y79 cells were cultured in Roswell Park Memorial Institute 1640 medium (Thermo Fisher Scientific). All the complete culture media were made by supplementation with 10% fetal bovine serum (Thermo Fisher Scientific) and penicillin-streptomycin (Thermo Fisher Scientific). All the cells were cultured at 37 ℃ under 21% O_2 and 5% CO_2. All the cells were authenticated by short tandem repeat profiling. Free of contamination of mycoplasma was checked by mycoplasma detection kit (Thermo Fisher Scientific).

Genetic Manipulation and Stable Cell Line Development Lentivirus plasmids, pCDH-CMV (cytomegalovirus)-MCS (multiple cloning site)-EF1 (elongation factor 1α)-Puro and pLKO-CMV-MCS-EF1-Puro, were kindly provided by Professor Hongbin Ji (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China), and were used for producing target gene-overexpressing and knockdown stable cells as described previously[13]. Briefly, pCDH-CMV-CRX-EF1-Puro and pCDH-CMV- transcription factor 7 (TCF7)-EF1-Puro plasmids were produced by inserting the coding sequence region of *CRX* and *TCF7*, amplified by polymerase chain reaction (PCR) with genomic DNA of RPE-1 cells, into pCDH-CMV-MCS-EF1-Puro plasmids, respectively. pLKO-CMV-shCRX#1/#2-EF1-Puro and pLKO-CMV-shTCF7#1/#2-EF1-Puro plasmids were developed by inserting shRNAs specific against *CRX* and *TCF7* into pLKO-CMV-MCS-EF1-Puro plasmids. Lentivirus plasmids were produced by co-transfection of recombinant plasmids together with packaging plasmid (pCMV-dR8.91) and envelope plasmid (pCMV-VSV-G) into HEK293T cells (Clontech, Japan) at a ratio of 5:4:1 using jetPEI (PolyPlus Transfection, France). The stable cell lines were established by infection of the cells with the lentivirus particles for 7d,

Table 1 shRNAs used in this study

shRNA	Sequence (5′-3′)
shCRX#1	GTGAAGATCTGGTTCCAAA
shCRX#2	GAAGGAGTTTCACTACAGT
shTCF7#1	CAACTCTCTCTCTACGAACAT
shTCF7#2	CCAAGAAGCCAACCATCAA
Scramble	CCGGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTG

followed by puromycin (Sigma, USA) selection. The efficiency of genetic manipulation was examined by both quantitative real-time polymerase chain reaction (qPCR) and western blot. The sequences of shRNAs were showed in Table 1.

Proliferation Assay ARPE-19 and RPE-1 cells were seeded at 5000 cells/100 μL in 96-well plate. Y79 cells were seeded at 3000 cells/100 μL in a 96-well plate. All the cells were cultured in the complete culture media for 48h. Cell proliferation was assessed at the endpoint with CellTiter-Glo Reagent [Promega; Cell proliferation=luminescence (endpoint–startpoint)/startpoint]. The luminescence was quantified with a plate reader.

Quantitative Real-Time Polymerase Chain Reaction Total RNAs from the cells were extracted by Trizol reagent (Thermo Fisher Scientific) according to the standard protocol. Nanodrop equipment was used for determining the concentration of the RNAs (Thermo Fisher Scientific, USA). qPCR was performed using SYBR green reagent (Thermo Fisher Scientific, USA) with an ABI stepOne real-time PCR system. The expression of the target genes was determined using the comparative Ct method with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. The sequences of the primers were showed in Table 2.

Western Blot The cells were first lysed in racial and identity profiling act (RIPA) buffer (Thermo Fisher Scientific). The proteins in lysates were separated by electrophoresis on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide gels) and transferred to polyvinylidene difluoride membrane (Thermo Fisher Scientific) by semidry blotting. The membranes were blocked for 1h at room temperature with 1%-3% bovine serum albumin (Thermo Fisher Scientific) or 5% nonfat dry milk (Thermo Fisher Scientific) in Tris-buffered saline (Thermo Fisher Scientific) containing 0.05% Tween-20 (Thermo Fisher Scientific). The membranes were then incubated overnight at 4℃ in blocking buffer (Thermo Fisher Scientific) containing the indicated primary antibodies. After washed in blocking buffer, the membranes were then incubated with the secondary antibodies conjugated to horseradish peroxidase. Visualization of the protein bands was performed using Clarity Western chemiluminescence (ECL) substrate chemiluminescent detection reagent (Bio-Rad, USA) according to the manual.

Dual-Luciferase Reporter Assay To determine the activation of Wnt signaling, a pGL4.49 [luc2P/TCF (T cell factor)-LEF (lymphoid-enhancer-binding factor)/Hygro] vector (Promega, USA) was transfected into the cells and the luciferase activity was examined according to the instruction. To determine the transcriptional activity of *TCF7* promoter and find the binding site of *CRX* in *TCF7* promoter, the promoter segments spanning -1200 to +115, -800 to +115, -400 to +115 bp relative to the transcription start site of *TCF7* were cloned into PGL4.20 plasmids (Promega). Next, the target cells were co-transfected with reconstructed luciferase reporter plasmids and hRluc/TK plasmids. After 48h culture, the cells were lysed, and luciferase activity was detected with Dual-Luciferases Reporter Assay kit (Promega). Relative firefly luciferase activity was normalized to renilla luciferase activity.

Nascent RNA Quantification The synthesis of TCF7, MYC proto-oncogene (MYC), Jun proto-oncogene (JUN), and cyclin D1 (CCND1) mRNAs was measured using Click-iT™ Nascent RNA Capture Kit (Thermo Fisher Scientific) according to manufacturer's instruction. Briefly, the freshly cultured cells were incubated in the culture medium containing 0.5 mmol/L 5-ethynyl uridine for 1h. EU-labeled RNA was extracted and mixed with biotin azide (10 μg RNA : 1 mmol/L Biotin Azide) for biotinylation. The biotinylated RNA was purified with a streptavidin-coupled magnetic beads, followed by measurement with qPCR.

Chromatin Immunoprecipitation Assay The direct binding between CRX protein and the promoter of TCF7 promotor was determined by chromatin immunoprecipitation (ChIP) assay with MAGnify™ Chromatin IP System (Thermo Fisher Scientific) according to manufacturer's instruction. Briefly, the cells were treated with 1% formaldehyde, followed by lysis. Chromatin in the cells was fragmentated by sonication and collected by centrifugation. CRX antibody-Dynabeads protein A/G were prepared for immunoprecipitation CRX-linked DNA. The precipitated DNA was examined by qPCR.

CRX Correlated Genes Analysis The gene expression data was downloaded from R2 bioinformatic database (https://r2.amc.nl/). The correlated genes of CRX were identified by Spearman analysis.

Statistical Analysis The data were represented as the mean ± standard deviation (SD) from three independent

Table 2 Primers used in this study

Gene	Sequence
Reverse transcription PCR	
CRX	5′-GGTACCATGTATGTGGGCTATGTGCTGGAC-3′
	5′-GAATTCTGGCAGAAACTCCTCTTTCACAG-3′
TCF7	5′-GAATTCGAGCACTGTCATCGGAAGGAAC-3′
	5′-GGTACCATGCCGCAGCTGGACTCC-3
Real-time quantitative reverse transcription	
CRX	F: 5′-GCCCCACTATTCTGTCAACG-3′
	R: 5′-GTCTGGGTACTGGGTCTTGG-3′
TCF7	F: 5′-CTGGCTTCTACTCCCTGACCT-3′
	R: 5′-ACCAGAACCTAGCATCAAGGA-3′
MYC	F: 5′-GGCTCCTGGCAAAAGGTCA-3′
	R: 5′-CTGCGTAGTTGTGCTGATGT-3′
JUN	F: 5′-TCCAAGTGCCGAAAAAGGAAG-3′
	R: 5′-CGAGTTCTGAGCTTTCAAGGT-3′
FOSL1	F: 5′-CAGGCGGAGACTGACAAACTG-3′
	R: 5′-TCCTTCCGGGATTTTGCAGAT-3′
CCND1	F: 5′-GCTGCGAAGTGGAAACCATC-3'
	R: 5'-CCTCCTTCTGCACACATTTGAA-3'
CCND2	F: 5′-ACCTTCCGCAGTGCTCCTA-3′
	R: 5′-CCCAGCCAAGAAACGGTCC-3′
CCND3	F: 5′-TACCCGCCATCCATGATCG-3′
	R: 5′-AGGCAGTCCACTTCAGTGC-3′
CCN4	F: 5′-GTGCTGTAAGATGTGCGCTCA-3′
	R: 5′-CCCGCTGTAGTCACAGTAGAG-3′
PPARD	F: 5′-CAGGGCTGACTGCAAACGA-3′
	R: 5′-CTGCCACAATGTCTCGATGTC-3′
MMP7	F: 5′-GAGTGAGCTACAGTGGGAACA-3′
	R: 5′-CTATGACGCGGGAGTTTAACAT-3′
DVL1	F: 5′-GAGGGTGCTCACTCGGATG-3′
	R: 5′-GTGCCTGTCTCGTTGTCCA-3′
DVL2	F: 5′-GAGGAAGAGACTCCCTACCTG-3′
	R: 5′-CGGGCGTTGTCATCTGAAAT-3′
DVL3	F: 5′-GACGCCGTACCTTGTGAAG-3′
	R: 5′-CGCTGCAAAACGCCCTTAAA3′
LEF1	F: 5′-AGAACACCCCGATGACGGA-3′
	R: 5′-GGCATCATTATGTACCCGGAAT-3′
CTNNB1	F: 5′-AAAGCGGCTGTTAGTCACTGG-3′
	R: 5′-CGAGTCATTGCATACTGTCCAT-3′
SENP2	F: 5′-GGCTGGTTAGGATTCTCGGC-3′
	R: 5′-GGCAGCATTGTAGAGACTGTTTT-3′
FRAT1	F: 5′-CCACGCCCTGTCTAAAGTGT-3′
	R: 5′-TGCAAAACACTGCGCTCTTC -3′
FRAT2	F: 5′-GCGGCTGGTAACTTCTCTGG-3′
	R: 5′-GGGAAACCTCATCAAGGAAGC-3′
CSNK1E	F: 5′-CGTGTGGGGAACAAGTACCG-3′
	R: 5′-GATGTTGGCACCCAGGTAGAT-3′
CCDC8	F: 5′-CAGCGACTTCGAGACCTCC-3′
	R: 5′-CAGGTAGCTGACGGGCATTT-3′
CXXC4	F: 5′-ATGCACCACCGAAACGACTC-3′
	R: 5′-GCAGTGTTCAGGGGATAAGGT-3′
AXIN1	F: 5′-GGTTTCCCCTTGGACCTCG-3′
	R: 5′-CCGTCGAAGTCTCACCTTTAATG-3′
CSNK1A1L	F: 5′-TCCAAAGCCGAACTCGTTGT-3′
	R: 5′-GATGCCCAGATAAACGTCTCC-3′
APC1	F: 5′-TCCTTCATCACAGAAACAGT-3′
	R: 5′-GCTGGATTTGGTTCTAGGG-3′
APC2	F: 5′-GGACCGGGAACGGTGTTTC-3′
	R: 5′-AGCTGAGAGTAGTACCAGAGC-3′
SOX17	F: 5′-GTGGACCGCACGGAATTTG-3′
	R: 5′-GGAGATTCACACCGGAGTCA-3′
CBY1	F: 5′-TCTTTGGGAATACGTTCAGTCCG-3′
	R: 5′-CCAGGTTCATAGTCGGGGA-3′
CSNK1A1	F: 5′-AGTGGCAGTGAAGCTAGAATCT-3′
	R: 5′-CGCCCAATACCCATTAGGAAGTT-3′
CTNNBIP1	F: 5′-CCTATGCAGGGGTGGTCAAC-3′
	R: 5′-CGACCTGGAAAACGCCATCA-3′
CHD8	F: 5′-TACAGCAGGCTCAGATAATGGG-3′
	R: 5′-TTGCTTCTTCTGGTGTTCCAAT-3′
RUVBL1	F: 5′-AGGTGAAGAGCACTACGAAGA-3′
	R: 5′-CTACTATGACGCCACATGCCT-3′
CREBBP	F: 5′-CAACCCCAAAAGAGCCAAACT-3′
	R: 5′-CCTCGTAGAAGCTCCGACAGT-3′
EP300	F: 5′-AGCCAAGCGGCCTAAACTC-3′
	R: 5′-TCACCACCATTGGTTAGTCCC-3′
CTBP1	F: 5′-AAAGCCCTCCGCATCATCG-3′
	R: 5′-AGACGGCAATGCCTAAATCCC-3′
CTBP2	F: 5′-ATCCACGAGAAGGTTCTAAACGA-3′
	R: 5′-CCGCACGATCACTCTCAGG-3′
TLE2	F: 5′-CGACGCCAAGGTTTGCTTC-3′
	R: 5′-GAGTGCCGTAATCGGAAATATCA-3′
TLE1	F: 5′-GAGTCCCTGGACCGGATTAAA-3′
	R: 5′-AATACATCACATAGTGCCTCTGC-3′
TLE3	F: 5′-TATCCGCAGGGCAGACATC-3′
	R: 5′-GTTTGCCAGCTTGTCGTACTC-3′
GAPDH	F: 5′-GGAGCGAGATCCCTCCAAAAT-3′
	R: 5′-GGCTGTTGTCATACTTCTCATGG-3′

PCR: Polymerase chain reaction.

experiments except where indicated. Statistical significance for comparison between two groups of samples showing a normal distribution (Shapiro-Wilk test for normality) was determined using the unpaired two-tailed Student's *t*-test. Statistical significance for comparison between two groups showing a no normal distribution was determined using a two-tailed Mann-Whitney nonparametric test. Statistical significance for comparison of more than two groups, one-way ANOVA with Bonferroni post-hoc test was used. $P<0.05$ was considered significant.

RESULTS

CRX Promotes the Proliferation of RPE and RB Cells

Although the expression of CRX in both RPE and RB cells was previously explored[8,4-13], whether CRX possesses the

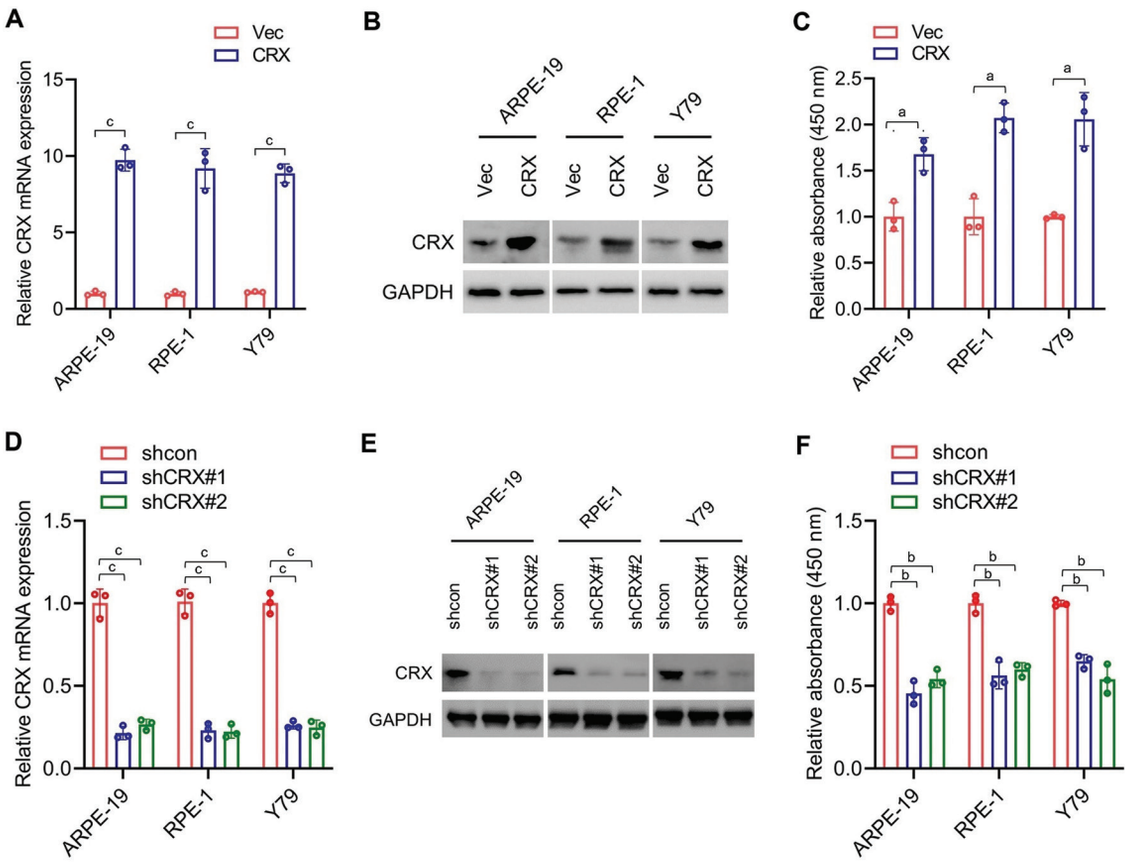

Figure 1 CRX promotes the proliferation of RPE and RB cells A, D: CRX mRNA expression levels analyzed by qPCR in CRX-overexpressing and empty vector control cells (n=3; A) or in CRX-knockdown and scrambled shRNA control cells (n=3; D). B, E: CRX protein expression levels analyzed by Western blot in CRX-overexpressing and empty vector control cells (n=3; B) or in CRX-knockdown and scrambled shRNA control cells (n=3; E). C, F: The proliferation of CRX-overexpressing and empty vector control cells (n=3; C) or CRX-knockdown and scrambled shRNA control cells (n=3; F) analyzed by CellTiter-Glo Reagent. ^{a}P<0.05; ^{b}P<0.01; ^{c}P<0.001, by unpaired, 2-tailed Student's t-test or one-way ANOVA. CRX: Cone-rod homeobox; Vec: Vector; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RPE: Retinal pigment epithelium; RB: Retinoblastoma.

ability to regulate the proliferation of RPE and RB cells has remained elusive. To study this, we employed two RPE cell lines, ARPE-19 and RPE-1, and one the RB cell line, Y79, as the cell model. The CRX-overexpressing stable cells were developed *via* a lentiviral-mediated gene delivery system. As shown in Figure 1A, 1B, the results from both qPCR and Western blot showed that CRX expression was increased more than 5-fold in CRX-overexpressing cells compared with control cells (transfected with the empty vectors; n=3, P<0.001 for qPCR assay in all the cells). We next examined the proliferation of CRX-overexpressing and control cells by CellTiter-Glo Reagent. As shown in Figure 1C, the proliferation was significantly increased in both CRX-overexpressing RPE and RB cells versus control cells (n=3, P<0.05 in all the cells), indicating the stimulatory effect of CRX on the proliferation of RPE and RB cells. To confirm this, the CRX was knocked down in ARPE-19, RPE-1, and Y79 cells. The efficiency of CRX knockdown was examined by qPCR and Western blot assays as well (Figure 1D, 1E; n=3, P<0.001 for qPCR assay in all the cells). As expected, the proliferation of ARPE-19, RPE-1 and Y79 cells was significantly reduced by CRX depletion (Figure 1F; n=3, P<0.01 in all the cells). These results suggested that CRX promotes the proliferation of both RPE and RB cells.

CRX Activates Wnt Signaling Pathway in RPE and RB Cells The Wnt signaling pathway plays fundamental roles in regulating the proliferation of various types of human cells, including RPE and RB[14-16]. By screening the correlated genes of CRX in RB specimens with The Cancer Genome Atlas (TCGA) dataset, we found that the expression of TCF7 and JUN (one of the transcription factors and target genes in Wnt signaling pathway, respectively) is positively correlated with CRX (Figure 2A; n=74, P<0.01 for TCF7 and P<0.05 for JUN). We, therefore, speculated that TCF7-mediated Wnt signaling is at least one of the downstream effectors of CRX in RPE and RB cells.

To investigate the effect of CRX on Wnt signaling pathway, we first employed qPCR to test the effect of CRX on the expression of the target genes in Wnt signaling pathway, including MYC proto-oncogene (MYC), JUN, FOS like 1 (FOSL1), CCND1, cyclin D2 (CCND2), cyclin D3 (CCND3),

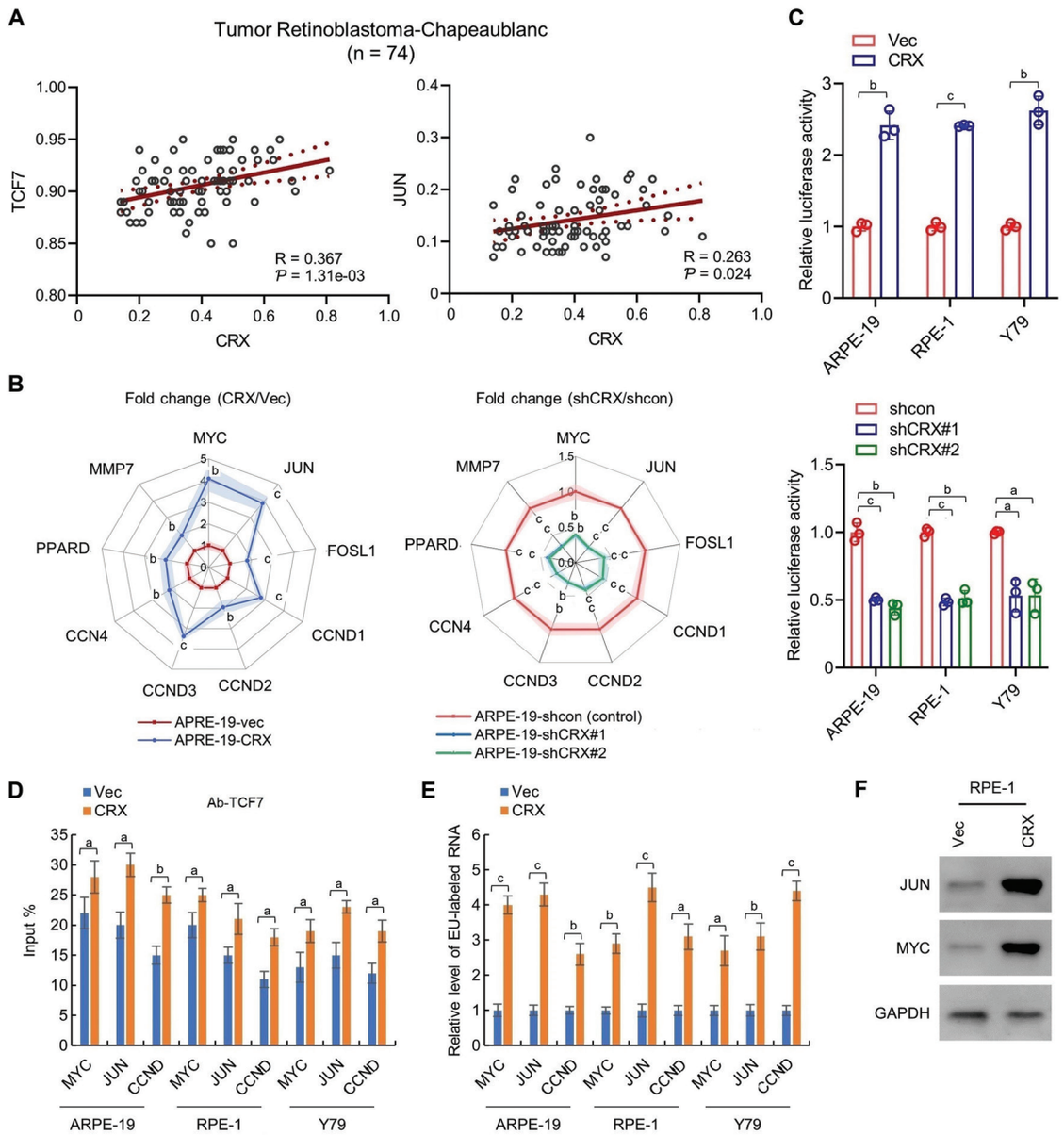

Figure 2 CRX promotes TCF7 transcriptional activity in RPE and RB cells A: Scatter plot shows the correlation between the mRNA levels of TCF7 or JUN and CRX in retinoblastoma tissues profiled by Chapeaublanc dataset downloaded from R2 online database. B: Radar plot shows that the mRNA levels of Wnt signaling target genes analyzed by qPCR in CRX-overexpressing and empty vector control cells (n=3) or in CRX-knockdown and scrambled shRNA control cells (n=3). C: The transcriptional activity of TCF/LEF analyzed by luciferase reporter assay in CRX-overexpressing and empty vector control cells (n=3) or in CRX-knockdown and scrambled shRNA control cells (n=3). D: ChIP-PCR analysis of the binding of TCF7 to the promoters of MYC, JUN, and CCND1 in CRX-overexpressing and empty vector control cells (n=3). E: The levels of nascent RNAs of *MYC*, *JUN*, and *CCND1* genes in CRX-overexpressing and empty vector control cells (n=3) analyzed by a modified nuclear run-on assay. F: Western blot analysis of the protein levels of JUN and MYC in CRX-overexpressing and empty vector control cells. ^{a}P<0.05; ^{b}P<0.01; ^{c}P<0.001, by unpaired, 2-tailed Student's t-test or one-way ANOVA. TCF7: Transcription factor 7; JUN: Jun proto-oncogene; MYC: MYC proto-oncogene; FOSL1: FOS like 1; CCND1: Cyclin D1; CCND2: Cyclin D2; CCND3: Cyclin D3; CCN4: Cellular communication network factor 4; PPARD: Peroxisome proliferator activated receptor delta; MMP7: Matrix metallopeptidase 7; CRX: Cone-rod homeobox; Vec: Vector; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; EU: 5-ethynyl uridine; TCF: T cell factor; LEF: Lymphoid-enhancer-binding factor; RPE: Retinal pigment epithelium; RB: Retinoblastoma.

cellular communication network factor 4 (CCN4), peroxisome proliferator activated receptor delta (PPARD), and matrix metallopeptidase 7 (MMP7), in ARPE-19 cell. The results showed that the mRNA levels of these genes were significantly increased in CRX-overexpressing APPE-19 cells (Figure 2B; n=3, P<0.01 for *MYC*, *MMP7*, *PPARD*, *CCN4*, and *CCND2*, and P<0.001 for the rest genes), and conversely, were significantly decreased in CRX-knockdown ARPE-19

cells (Figure 2B; n=3, P<0.01 for MYC and CCND3 in both shCRX#1 and shCRX#2 cells; P<0.001 for MMP7, PPARD, CCN4, CCND2, CCND1, and FOSL1 in both shCRX#1 and shCRX#2 cells), indicating the stimulatory effect of CRX on Wnt signaling pathway in RPE and RB cells. Similarly, we transfected TCF/LEF luciferase reporter plasmids into CRX-overexpressing and control ARPE-19, RPE-1, and Y79 cells and found that the luciferase activities were significantly increased in CRX-overexpressing cells versus empty vector control cells (Figure 2C; n=3, P<0.01 in ARPE-19 and Y79 cells, P<0.001 in RPE-1 cell), and oppositely, were significantly decreased in CRX-knockdown cells versus control cells (Figure 2C; n=3, P<0.001 and 0.01 for shCRX#1 and shCRX#2 in ARPE-19 cells, P<0.001 and 0.01 for shCRX#1 and shCRX#2 in RPE-1 cells, P<0.05 for both shCRX#1 and shCRX#2 in Y79 cells), which indicated that CRX promotes the transcriptional activity of the TCF/LEF transcription factors in Wnt signaling pathway in RPE and RB cells. In addition, the result from the ChIP-PCR assay showed that the DNA levels of the promoters of MYC, JUN, and CCND1 immunoprecipitated by an antibody specifically against TCF7 were significantly increased in CRX-overexpressing cells versus control cells (Figure 2D; n=3, P<0.05 for MYC and 0.001 for JUN, and CCND1 in ARPE-19 cells, P<0.05 for all in RPE-1, and Y79 cells), indicating that CRX promotes the binding between TCF7 and its targets in RPE and RB cells. Moreover, by a modified nuclear run-on assay, increased nascent RNA levels of MYC, JUN, and CCND1 in CRX-overexpressing cells versus control cells were observed (Figure 2E; n=3, P<0.001, 0.001, 0.01 for MYC, JUN, and CCND1 in ARPE-19 cells, P<0.01, 0.001, 0.05 for MYC, JUN, and CCND1 in RPE-1 cells, P<0.05, 0.01, 0.001 for MYC, JUN, and CCND1 in Y79 cells), which indicated that CRX promotes the transcription of Wnt signaling target genes in RPE and RB cells. Furthermore, overexpression of CRX led to increased protein levels of MYC and JUN in RPE-1 cells as identified by western blot (Figure 2F). Taken together, these results demonstrated that CRX activates Wnt signaling pathway in RPE and RB cells.

TCF7 Promotes the Proliferation of RPE and RB Cells The effect of TCF7 on the proliferation in RPE and RB cells has remained elusive. To further confirm that CRX promotes the proliferation of RPE and RB cells through TCF7-mediated Wnt signaling pathway, we next examined the regulatory effect of TCF7 on the proliferation in RPE and RB cells. The TCF7-overexpressing and TCF7-knockdown stable ARPE-19, RPE-1, and Y79 cells were developed. The overexpression of TCF7 in ARPE-19, RPE-1, and Y79 cells was demonstrated by both qPCR and Western blot (Figure 3A, 3B; n=3, P<0.01 for qPCR assay in APRE-19 cell, P<0.01 for qPCR assay in RPE-1 and Y79 cells). As expected, overexpression of TCF7 led to increased proliferation in ARPE-19, RPE-1, and Y79 cells versus empty vector control cells (Figure 3C; n=3, P<0.01 in APRE-19 cell, P<0.001 in RPE-1 and Y79 cells). The opposite results were observed in TCF7-knockdown ARPE-19, RPE-1, and Y79 cells. The efficiency of TCF7-knockdown was demonstrated by qPCR and Western blot (Figure 3D, 3E; n=3, P<0.01 for both shTCF7#1 and shTCF7#2 in APRE-19 and Y79 cells; P<0.05 for both shTCF7#1 and shTCF7#2 in RPE-1 cell). The proliferation ability was significantly decreased in TCF7-knockdown cells versus control cells (Figure 3F; n=3, P<0.001 for both shTCF7#1 and shTCF7#2 in all the cells). These results demonstrated that TCF7 promotes the proliferation of RPE and RB cells.

CRX Promotes Wnt Signaling Pathway and Proliferation in RPE and RB Cells Through TCF7 Next, we asked whether TCF7 was the only downstream effector of CRX in Wnt signaling pathway in RPE and RB cells. As shown in Figure 4A, only TCF7 showed a significant increase in the mRNA level in CRX-overexpressing ARPE-19, RPE-1, and Y79 cells (n=3, P<0.001 in all the cells). A similar result was observed in CRX-knockdown cells that only TCF7 showed a significant decrease in the mRNA level in CRX-knockdown ARPE-19, RPE-1, and Y79 cells (Figure 4B; n=3, P<0.001 in all the cells). Furthermore, the increased protein levels of CRX were observed in CRX-overexpressing cells versus control cells as identified by western blot (Figure 4C). These results demonstrated that TCF7 is the only target in Wnt signaling in RPE and RB cells, at least at the transcriptional level.

Next, to confirm that TCF7 mediates the effect of CRX on Wnt signaling and the proliferation in RPE and RB cells, we depleted TCF7 in CRX-overexpressing ARPE-19, RPE-1, and Y79 cells (Figure 4D). As expected, TCF7 depletion at least partially abolished the stimulatory effect of CRX on the proliferation of ARPE-19, RPE-1, and Y79 cells (Figure 4E; n=3, CRX-shTCF7 *vs* CRX-shcon, P<0.01 in ARPE-19 and Y79 cells and P<0.001 in RPE-1 cell). In addition, the mRNA levels of Wnt target genes in CRX-overexpressing cells were partially recovered by TCF7 depletion (Figure 4F, n=3, CRX-shTCF7 *vs* CRX-shcon, P<0.01 for *MYC*, *MMP7*, *CCND1*, *CCND2*, and *FOSL1*, and P<0.001 for rest in ARPE-19 cell; P<0.01 for *JUN* and *FOSL1*, P<0.001 for rest in RPE-1 cells; P<0.01 for *MYC*, *CCN4*, and *CCND1*, and P<0.001 for rest in Y79 cells). These results demonstrated that TCF7 mediates the effect of CRX on Wnt signaling and the proliferation in RPE and RB cells.

CRX Directly Binds to the Promoter of TCF7 Considering CRX normally functions as a transcription factor, to investigate the mechanism underlying CRX regulating the transcription of TCF7, we employed ChIP-PCR to identify the direct binding between CRX and the promoter of TCF7. We found that the

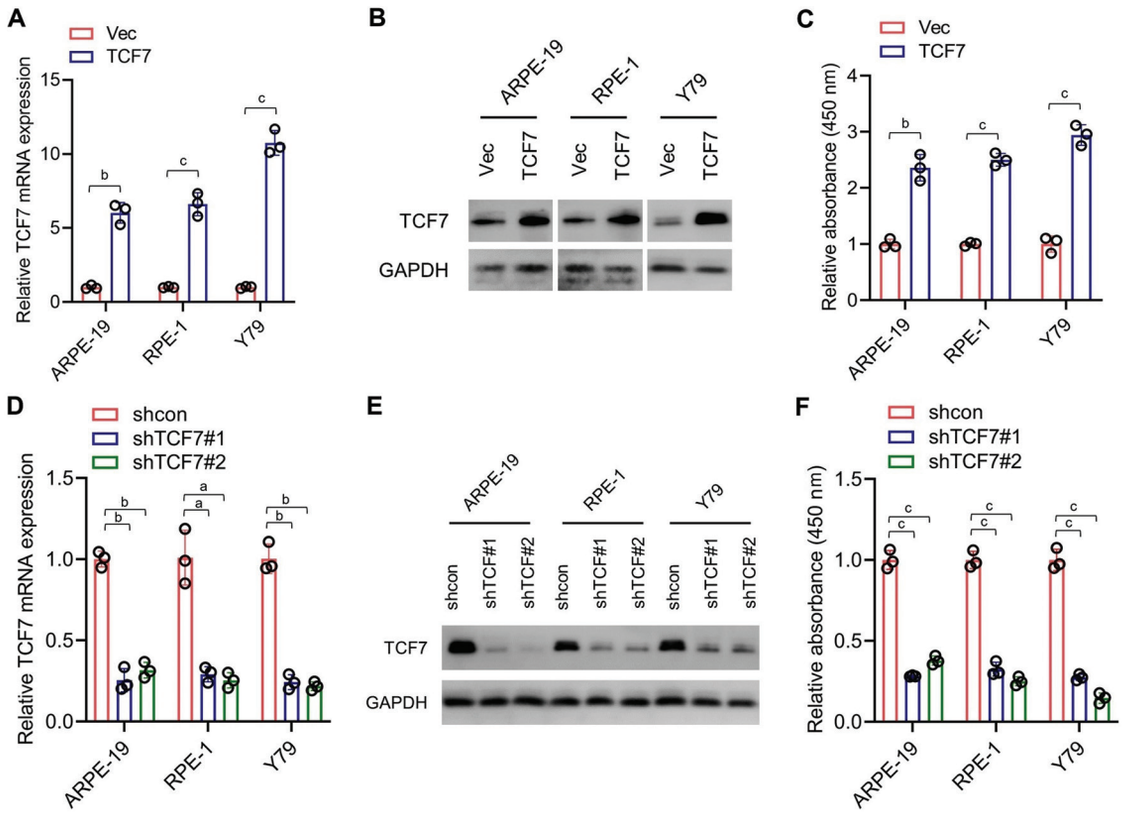

Figure 3 TCF7 promotes the proliferation of RPE and RB cells A, D: TCF7 mRNA expression levels analyzed by qPCR in TCF7-overexpressing and empty vector control cells (n=3; A) or in TCF7-knockdown and scrambled shRNA control cells (n=3; D). B, E: TCF7 protein expression levels analyzed by Western blot in TCF7-overexpressing and empty vector control cells (n=3; B) or in TCF7-knockdown and scrambled shRNA control cells (n=3; E). C, F: The proliferation of TCF7-overexpressing and empty vector control cells (n=3; C) or TCF7-knockdown and scrambled shRNA control cells (n=3; F) analyzed by CellTiter-Glo Reagent. ^{a}P<0.05; ^{b}P<0.01; ^{c}P<0.001; by unpaired, 2-tailed Student's t-test or one-way ANOVA. TCF7: Transcription factor 7; Vec: Vector; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TCF: T cell factor; ARPE: Adult human retinal pigment epithelial; RPE: Human retinal pigment epithelial; RB: Retinoblastoma.

promoter of TCF7 could be successfully immunoprecipitated by the antibody specifically against CRX, and the volumes were significantly higher in CRX-overexpressing cells versus control cells (Figure 5A; n=3, P<0.05 in all the cells). Moreover, through a modified nuclear run-on assay, we showed that the transcription of TCF7 was significantly upregulated in CRX-overexpressing ARPE-19, RPE-1, and Y79 cells (Figure 5B; n=3, P<0.05 in RPE-1 cell and P<0.01 in APRE-19 and Y79 cells). Furthermore, by luciferase reporter assay, we found that -1200 to -400 bp in TCF7 promoter responses CRX in ARPE-19, RPE-1, and Y79 cells (Figure 5C). These results demonstrated that CRX directly binds to the promoter of TCF7 and promotes the transcription of TCF7. Collectively, the above results provide *in vitro* evidence supporting a novel regulatory mechanism involved in the proliferation of RPE and RB cells that CRX directly binds to the promoter of TCF7, stimulates TCF7 transcription, activates Wnt signaling pathway, and thereby promoting the proliferation of RPE and RB cells (Figure 5D).

DISCUSSION

In this study, we revealed that CRX promotes the proliferation of RPE and RB cells through TCF7-meiwadated Wnt signaling pathway. Our findings suggested that CRX was a potential target for RPE-based regenerative medicine; however, the potential risk of this strategy, tumorigenic potential, should be considered.

Dysregulation of RPE causes various eye-related disorders. For example, mutations of genes, especially those involved in the retinoid cycle, causes inherited multifocal RPE diseases, including Stargardt disease, retinol binding protein deficiency syndrome, fundus albipunctatus, and retinitis punctata albescens[17]. Furthermore, the well-known inherited eye disorder, CRD, can also be the result of mutation of genes involved in the degeneration of RPE cells; Parry *et al*[18] revealed that, in 12-month-old mice, depletion of metallopeptidase domain 9 (*ADAM9*) gene, identified in four consanguineous families with recessively inherited early-onset CRD, caused disorganization of RPE cells but not photoreceptors. These finds suggest RPE was an important therapeutic target for eye diseases involved in not only RPE itself, but also photoreceptors. Meanwhile, recent studies involved in proliferative potential of RPE cells[6-7] yield a

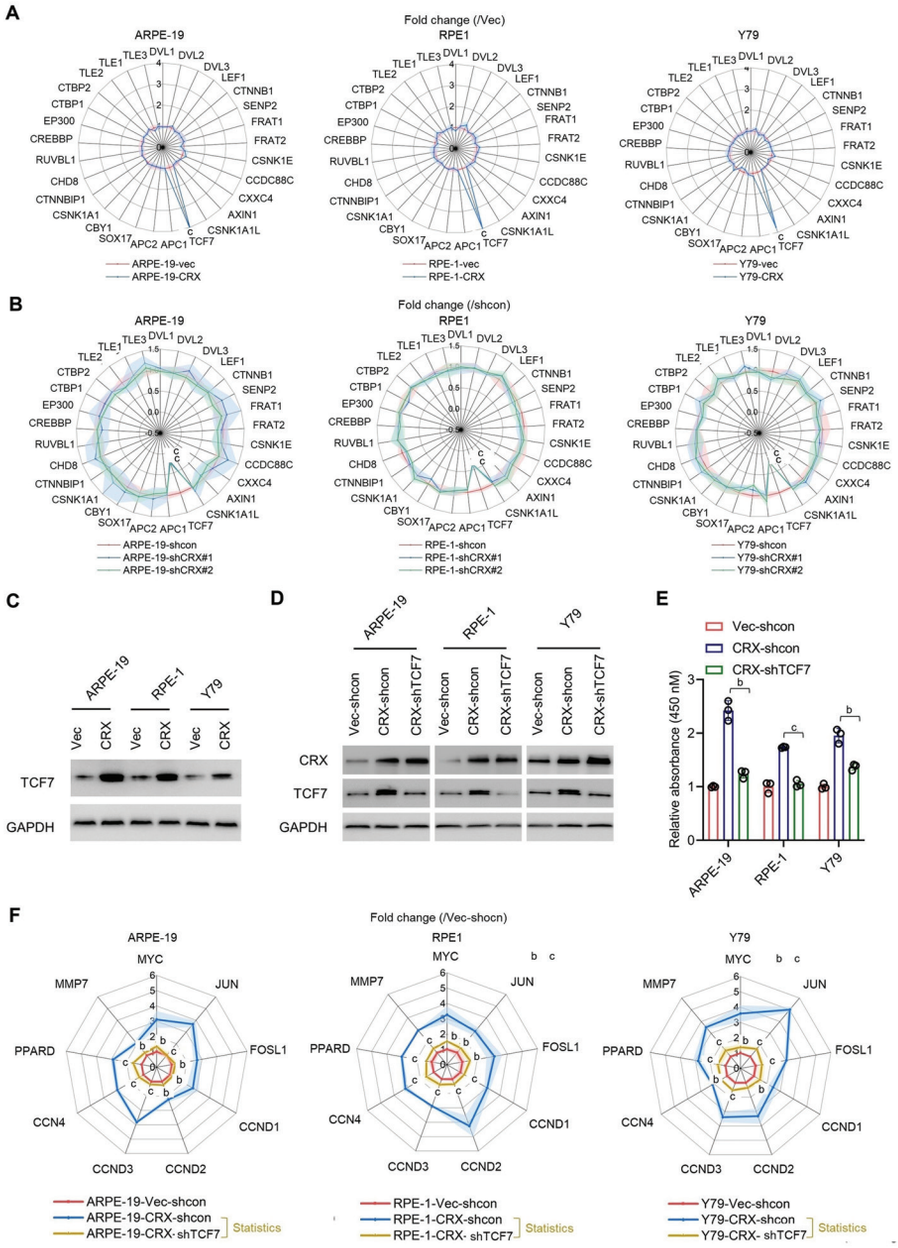

Figure 4 TCF7 is critical for CRX promoting the Wnt signaling pathway and the proliferation in RPE and RB cells A, B: Radar plot shows that the mRNA levels of key components in Wnt signaling pathway analyzed by qPCR in CRX-overexpressing and empty vector control cells (*n*=3; A) or in CRX-knockdown and scrambled shRNA control cells (*n*=3; B). C: Western blot analysis of the protein level of TCF7 in CRX-overexpressing and empty vector control cells. D: Characterization of CRX-overexpressing TCF7-knockdown cells by Western blot analysis. E: The proliferation of CRX-overexpressing TCF7-knockdown cells analyzed by CellTiter-Glo Reagent (*n*=3). F: Radar plot shows the mRNA levels of Wnt signaling target genes in CRX-overexpressing TCF7-knockdown cells analyzed by qPCR (*n*=3). [a]*P*<0.05; [b]*P*<0.01; [c]*P*<0.001, by unpaired, 2-tailed Student's *t*-test or one-way ANOVA. JUN: Jun proto-oncogene; MYC: MYC proto-oncogene; FOSL1: FOS like 1; CCND1: Cyclin D1; CCND2: Cyclin D2; CCND3: Cyclin D3; CCN4: Cellular communication network factor 4; PPARD: Peroxisome proliferator activated receptor delta; MMP7: Matrix metallopeptidase 7; CRX: Cone-rod homeobox; Vec: Vector; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; EU: 5-ethynyl uridine; DVL1: Dishevelled segment polarity protein 1; DVL2: Dishevelled segment polarity protein; DVL3: Dishevelled segment polarity protein 3; LEF1: Lymphoid enhancer binding factor 1; CTNNB1: Catenin beta 1; SENP2: SUMO specific peptidase 2; FRAT1: FRAT regulator of WNT signaling pathway 1; FRAT2: FRAT regulator of WNT signaling pathway 2; CSNK1E: Casein kinase 1 epsilon; CCDC88C: Coiled-coil domain containing 88C; CXXC4: CXXC finger protein 4; AXIN1: Axin 1; CSNK1A1L: Casein kinase 1 alpha 1 like; TCF7: Transcription factor 7; APC1: Anaphase promoting complex subunit 1; APC2: Anaphase promoting complex subunit 2; SOX17: SRY-box transcription factor 17; CBY1: Chibby 1, beta catenin antagonist; CSNK1A1: Casein kinase 1 alpha 1; CTNNBIP1: Catenin beta interacting protein 1; CHD8: Chromodomain helicase DNA binding protein 8; RUVBL1: RuvB like AAA ATPase 1; CREBBP: CREB binding protein; EP300: E1A binding protein p300; CTBP1: C-terminal binding protein 1; CTBP2: C-terminal binding protein 2; TLE1: TLE family member 1, transcriptional corepressor; TLE2: TLE family member 2, transcriptional corepressor; TLE3: TLE family member 3, transcriptional corepressor; TLE: Transducin-like enhancer of split family proteins.

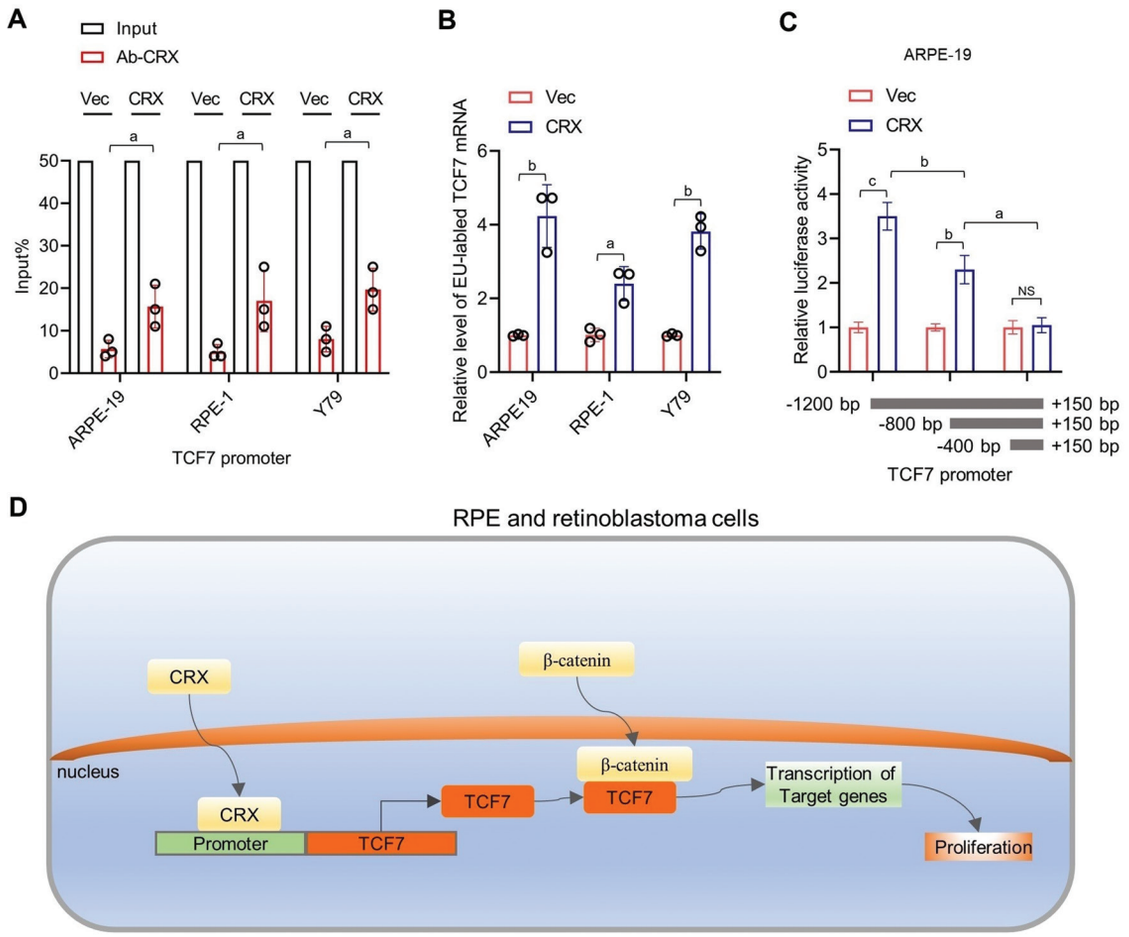

Figure 5 CRX directly binds to the promoter of TCF7 A: ChIP-PCR analysis of the binding of CRX to the promoters of *TCF7* in CRX-overexpressing and empty vector control cells (*n*=3). B: The levels of nascent RNAs of *TCF7* in CRX-overexpressing and empty vector control cells analyzed by a modified nuclear run-on assay (*n*=3). C: The transcriptional activity of different fragments of TCF7 promoter in CRX-overexpressing and empty vector control cells analyzed by luciferase reporter assay (*n*=3). D: The proposed mechanism by which CRX transcriptionally activates TCF7, strengthen Wnt signaling, thereby promoting RPE cell proliferation. $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$, by unpaired, 2-tailed Student's *t*-test or one-way ANOVA. TCF7: Transcription factor 7; CRX: Cone-rod homeobox; Vec: Vector; RPE: Retinal pigment epithelium; EU: 5-ethynyl uridine; ARPE: Adult human retinal pigment epithelial.

potential therapeutic opportunity for human diseases caused by the degeneration of RPE cells.

In our study, *CRX* has been identified as a positive proliferation regulatory gene in RPE cells (Figure 1). Subsequent studies demonstrated that CRX promoted the proliferation of RPE cells by activating TCF7-mediated Wnt signaling (Figures 2-5). The Wnt signaling pathway is an important pathway that is associated with a variety of key cellular functions in mammalian cells[19]. In this pathway, Wnt-stabilized β-catenin translocates to the nucleus and interacts with T cell factor/lymphoid-enhancer-binding factor (TCF/LEF) family transcription factors to promote the transcription of target genes[20]. Wnt signaling is also essential for RPE development and RPE derivation from human embryonic stem cells[21-22]. These studies confirmed that *CRX* could be used as a drug target for RPE-based regenerative medicine.

Several studies have investigated the mechanisms underlying RPE cell survival and proliferation. For example, Zhu *et al*[23] found that inhibition of epidermal growth factor receptor (EGFR) attenuated the proliferation and migration of RPE cells *via* EGFR/AKT signaling pathway. Zhou *et al*[24] found that hepatocyte growth factor (HGF) was able to promote RPE cell proliferation and migration. Mao *et al*[25] reported that all-trans retinoic acid (ATRA) could regulates the expression of MMP-2 and transforming growth factor beta 2 (TGF-β2) in RPE cells. However, although these studies, the potential side effects of RPE-based regenerative medicine, such as oncogenic potential, have not be considered. We found that CRX also promoted the proliferation of RB cells by employing the mechanism as same as in RPE cells (Figures 1-5), which suggested the oncogenic potential of CRX-based regenerative medicine for RPE-based disorders.

On the other side, studies have been revealed the aberrant elevation of CRX in RB[26-27], however, the exact evidence

supporting the proliferation regulatory effect of CRX in RB cells was not obtained. Thus, the findings in our study provided the *in vitro* cell-based evidence for the proliferation stimulatory role of CRX in RB, and thus suggested a novel mechanism and drug target for RB.

In summary, our study demonstrated that CRX could be served as a potential drug target for RPE-based regenerative medicine, that promotes the proliferation of RPE and RB cells through TCF7-mediated Wnt signaling pathway. However, the potential risk of this strategy, tumorigenic potential, should be considered.

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Authors' contributions: Zhao N and Lang TY conceptualized the project and research plan. Li YY, Xu JM, Li YZ and Lang TY performed the molecular-based assay. Lu QK, Lam TC, Yang MY, and Zhou L designed the bioinformatic analysis pipeline and performed the bioinformatic analysis. Li YZ, Tong QH, Zhang JT, Wang SZ, Hu XX, and Wu YF performed the proliferation assay and analyzed the results. Zhao N and Lang TY supervised the project and wrote the manuscript.

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