Retinoblastoma (Rb) promotes senescence of corneal endothelial cells by inhibiting the activation of E2F2

Alan Jiang
Third Affiliated Hospital of Nanchang University
longbing Mao
Third Affiliated Hospital of Nanchang University
sujuan Duan
Third Affiliated Hospital of Nanchang University
yanyan Zhang
Third Affiliated Hospital of Nanchang University
xing Liu
Third Affiliated Hospital of Nanchang University
wenwen Cui
Third Affiliated Hospital of Nanchang University
yi Sang
Third Affiliated Hospital of Nanchang University
Guofu Huang (hgf2222@sina.com)

Research article

Keywords: Retinoblastoma (Rb), Senescence, Rabbit corneal endothelial cells, E2F2

Posted Date: May 31st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-23760/v1

Abstract

Background Senescence-like changes occur in aging Corneal endothelial cells (CECs), and these changes are associated with decreased vision and age-related corneal diseases. such as Fuchs endothelial dystrophy (FED), chronic corneal allograft dysfunction (CCAD). Such changes have also been shown to cultured cell in vitro after passaging. Therefore, studying the mechanism of CEC senescence would aid in the development of anti-senescence treatment, which would benefit FED and CCAD. The tumor suppressor retinoblastoma (RB) gene product pRB triggers senescent growth arrest when inactivated. In this study, we used siRNA treatment to evaluate whether RB knockdown could suppress CEC senescence, and we investigated relevant molecular mechanisms.

Methods RCECs were obtained and were cultured with or without siRb. Senescent cells were detected with a β-galactosidase senescence staining kit. The gene p21, which is associated with a senescent phenotype, was measured by RT-PCR. The morphology and migration of cultured RCECs were examined by phase-contrast microscopy. ZO-1 and N-Cadherin, which are involved in pump and barrier functions, were assessed by immunofluorescence. Cell cycle assessment was performed using a flow cytometer (BD FACSCalibur).

Results As the cells were passaged, the number of senescent RCECs, the levels of the senescence-related gene p21, and the levels of senescence-associated secretory factors increased. SiRNA-mediated knockdown of RB led to suppression of cell senescence and the SASP. Furthermore, RB intervention increased the numbers of cells at the G2/M and S phase but did not influence the cell function or migratory ability. Knockdown of RB promoted the activation of E2F2.

Conclusions We demonstrated that as the cells increased in passage number, the number of senescent RCECs, the levels of the senescence-related genes p21, and the levels of senescence-associated secretory factors increased. Retinoblastoma (Rb) promoted the senescence of corneal endothelial cells by inhibiting the activation of E2F2.

1. Background

Corneal endothelial cells (CECs) form a single layer situated in the back of the cornea.[1, 2] These cells have a pivotal function in maintaining corneal transparency by performing pump and barrier functions.[3] Because of cell-cell contacts and a lack of relevant growth factors, CECs are arrested in the G1 phase, and their proliferation ceases.[4] The relative number of CECs is approximately 3500–4000 cells/mm^2 at birth, and the number gradually decreases with age or following surgical trauma.[5] When the cell density falls to 500–1000 cells/mm^2, loss of corneal endothelial function results in visual loss.[6, 7]

Cell aging is a special form of persistent cell cycle arrest, and it is regulated by a number of biological events.[8] Senescence-like changes take place in aging CECs and are associated with decreased vision and age-related corneal diseases.[2] Fuchs endothelial dystrophy (FED)[9] manifests as corneal epithelial edema and blindness, and it results in upregulated expression of senescence-related proteins.[10, 11]

Chronic corneal allograft dysfunction (CCAD)[9] has become the main problem of clinical penetrating keratoplasty, and it leads to corneal allograft failure. Previous results have indicated that cellular senescence is associated with the pathogenesis of chronic graft failure.[12, 13] Tissue engineering to produce corneas has become a popular research area in recent years, but the availability of seed cells has greatly limited this development.[14–16] Several research groups have successfully cultured corneal endothelial cells. However, maintaining cultured CECs with the correct morphology and function is another problem. Moreover, as the cells are passaged, the cell density decreases, which was found to be associated with cellular senescence.[17–19] Therefore, analyzing the aging mechanism of CECs will aid in the development of anti-senescence treatments that will benefit FED and CCAD suppresses cellular senescence.

Cellular senescence is a special, irreversible state of cell cycle arrest. It is characterized by a series of features, including upregulated expression of aging-related proteins (e.g., $p16^{INK4a}$ and p21), and it exhibits a “senescence-associated secretory phenotype” (SASP), which includes the release of pro-inflammatory cytokines and chemokines.[20–22] A series of factors that induce stress, including DNA lesions or reactive oxygen species (ROS), have been shown to induce cellular senescence.[23, 24] Both the p53-p21-RB and $p16^{INK4a}$-RB effector pathways can activate growth arrest, ultimately leading to sustained senescence.[25] Furthermore, the tumor suppressor RB gene product pRB triggers senescent growth arrest when inactivated.[26] Further, activated E2F is tightly bound to pRB and prevents E2F activation and DNA synthesis.[1] Then, pRB is hyperphosphorylated by the cyclin D/CDK4 complex, which alters the interaction between pRB and E2F. [27, 28] Therefore, we hypothesized that the knockdown of RB would decrease this interaction and restrain cellular senescence.

In this study, we evaluated whether RB knockdown could suppress CEC senescence and investigated relevant molecular mechanisms.

2. Methods

2.1 Animals

Twenty New Zealand white rabbits in total (Experimental Animal Center of Nanchang University, Nanchang, Jiangxi, China; weight, 1.52.0 kg) were used in our study. Rabbits were housed and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experiments were approved by the university’s Animal Care and Use Committee.

2.2 Cell culture

All rabbits were placed in restraint devices under general anesthesia, followed by a lethal ear venous injection with 100 mg/kg of sodic pentobarbital. the eyeballs were excised and incubated in D-PBS containing 2% penicillin-streptomycin. Each rabbit corneal endothelium with Descemet's membrane was dissected after the cornea was separated under a stereoscopic dissecting light microscope. Corneas were washed three times with D-PBS containing 1% penicillin-streptomycin. The corneal endothelium, which

was attached to Descemet's membrane, was incubated overnight at 37 °C in DMEM containing 10% fetal bovine serum (FBS; Gibco-Life Technologies) and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA). The next day, the tissue was washed with D-PBS and digested at 37 °C for approximately 10–15 minutes in a Trypsin-EDTA solution. The resulting RCECs were suspended and cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin for 5 to 7 days until they were confluent, and then the cells were passaged at a 1:3 ratio using Trypsin-EDTA solution.

2.3 Gene silencing by siRNA transfection

The sequences of siRNA oligos targeting RB were as follows: siRB1, 5'- GCAUUACUCAAAGAACCAUTT – 3' (sense); 5'- AUGGUUCUUUGAGUAAUGCTT – 3' (anti-sense); siRB2, 5'- GCUUUCAGAUUCACCUUUATT – 3' (sense); and 5'-UAAAGGUGAAUCUGAAAGCTT-3' (anti-sense). When cells were plated at approximately 40% confluence, the medium was replaced with serum-free OPTI-MEM, and the cells were transfected for 8 hours using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, #13778030) according to the manufacturer's instructions. Then, the transfection reagent was removed, and the cells were cultured in DMEM for 48 hours. Rb expression levels were measured by RT-PCR.

2.4 Cell cycle analysis by propidium iodide (PI) staining

Cells transfected with siRNAs were maintained for 48 hours, and then they were suspended and

fixed with 70% ethyl alcohol overnight at -20 °C or at 4 °C for 1 hour for further experiments. Briefly, cells were centrifuged and washed with PBS. Subsequently, the cells were resuspended in PBS with propidium iodide (PI; 50 μg/ml, Sigma) and RNaseA (100 μg/ml, Beyotime), and then they were treated for 20 minutes at room temperature. Cell cycle analysis was performed immediately thereafter using a flow cytometer (BD FACSCalibur).

2.5 Real-time polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described.[29] In brief, total RNA was extracted by TRIzol reagent (Invitrogen, #15596026)from each sample 4 days after siRNA transfection, and cDNA was obtained using a PrimeScript™ RT reagent kit (Takara, #RR037B) according to the manufacturer's protocol. All RT-PCR was performed with SYBR GREEN reagents with a CFX96 Real-Time System (BIO-RAD, CFX96 optics module). The primer sequences used for RT-PCR analysis in our study are shown in Table 1. The resulting expression values were analyzed using the $2^{-\Delta\Delta CT}$ method.

Table 1
Primer sequences used for RT-PCR analysis in our study.

primers	Oligonucleotide
GAPDH	GCACCGTCAAGGCTGAGAAC (Forward)
GAPDH	TGGTGAAGACGCCAGTGGA (Reverse)
RB	TGGGAGAAAGTATCATCTGTGGA (Forward)
RB	TAAAAGTGAATGGCATCTCATCG (Reverse)
P21	ACGTGGACCTGTCGCTGACCTGC (Forward)
P21	GAAGACGAGTCGGCGTTTGGAGTG (Reverse)
IL-5	TCTTGGAGCTGCCTATGTTTGTG (Forward)
IL-5	ATTGCCTATCAGCAGACTCTGGTAAGT (Reverse)
IL-6	TCCTGGTGGTGGCTACCGCTTTC (Forward)
IL-6	TCTTTCCTCAGCTCCTTGATGGTCT (Reverse)
IL-8	ATGAACTCCAAGCTGGCTGTGGC (Forward)
IL-8	ATGCACTGGCATCGAAGCTCTGT (Reverse)
IL-1α	GTACCTCAGGGCAGCTCCACTAC (Forward)
IL-1α	TCATCTTCATTCTGGGCACTCAC (Reverse)
IL-1β	GTCTTGTCAGTCGTTGTGGCTCT (Forward)
IL-1β	CTGTAGTCATCCCAGGTGTTGCA (Reverse)
CCL2	GAAGAATCAACAGCACCAAGTGT (Forward)
CCL2	TTGGGTTGTGGAATAAGAGGTCA (Reverse)
TNF	CGCTCTTCTGCCTGCTGCACTTC (Forward)
TNF	CCACTTGCGGGTTTGCTACTACG (Reverse)
CXCL10	GAACTGTACGCTGTACCTGTATC (Forward)
CXCL10	TCCTCTGGACCTTTCCTTGCTAA (Reverse)
MIF	AGCTTGCACAGCATCGGCAAGATC (Forward)
MIF	GTTGGCCGCATTCATGTCGAAGTAG (Reverse)
E2F2	GGCGCATCTACGACATCACCAACG (Forward)
E2F2	TCAGCTCCTTCAGCTCCTGCCTCA (Reverse)
E2F4	GGCTTCTTACCACCAAGTTCGTGTC (Forward)

primers	Oligonucleotide
E2F4	GATCAGCTTGTCGGCAATCTCCC (Reverse)

2.6 β-Galactosidase Senescence Staining

Senescent cells were detected with a cell senescence β-galactosidase staining kit (Beyotime, C0602) according to the protocol provided by the manufacturer. First, each cell sample was washed with PBS once, and then it was fixed with β-galactosidase fixing solution buffer for 10 minutes. After that, the cells were washed three times with PBS for 3 minutes and incubated at 37 °C overnight with a staining buffer containing X-gal. Samples were visualized the next day using a fluorescence inverted microscope (Olympus, IX73).

2.7 Immunofluorescent Staining

Immunofluorescent staining was used to assess the expression of ZO-1 and N-cadherin, which are involved in pump and barrier functions. Briefly, confluent RCECs were fixed with a 4% paraformaldehyde solution, which was followed by blocking with 1% bovine serum albumin (BSA) in PBS for 1 hour; then, cells were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes. Each sample was rinsed 3 times with PBS for 5 minutes each time. Cells were incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used in our study: ZO-1 (diluted 1:100; Thermo Fisher Scientific, #33-9100) and N-cadherin (diluted 1:100; Thermo Fisher Scientific, #MA1-2002). After washing, the cells were probed with one of the following secondary antibodies at a 1:1000 dilution: Alexa Fluor 488-conjugated goat anti-mouse (Life Technologies) or Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Life Technologies). Cells were stained with 4’,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 5 minutes. Finally, cells were imaged using an inverted fluorescence microscope (Olympus, IX73).

2.8 Statistical Analysis

We used Prism 6 software for statistical analysis. All results are shown as the means ± SD, and all data were analyzed via Student’s t-test. P values < 0.05 were considered statistically significant.

3. Results

3.1 Corneal endothelial cell senescence and senescence-associated secretion of factors throughout the passaging of cells

We initially compared the size and morphology of rabbit corneal endothelial cells at passages 1–5. As the passage number increased, the RCECs gradually became larger, the cell morphology gradually shifted from being hexagon to irregular, and the cell density showed a decline (Fig. 1a and b). The senescence marker SA-β-Gal, which was detected with a senescence detection kit, was used to evaluate the senescence of RCECs; the percentage of SA-β-gal-positive RCECs increased with cell passage (Fig. 1c and

d). Furthermore, RT-PCR experiments were performed to measure the expression of the senescence-related gene p21 and to measure secreted factors associated with SASP. Compared with P1, the expression of the senescence-related gene p21 increased at the mRNA level (Fig. 1e). Moreover, the expression of 7 secreted factors (CCL2, CXCL10, TNF, IL-1α, IL-1β, IL-6, and IL-8) was significantly upregulated in P3 (Fig. 1f). These data show that corneal endothelial cells gradually senesce throughout culture.

3.2 Effect of siRB on the senescence of RCECs

To investigate the effect of RB on RCEC senescence, we used siRNAs to knock down RB in P3 cells, which is a passage of cells that exhibits qualities of cellular senescence but is in a good cellular state. RB mRNA levels were downregulated following siRNA treatment, as shown by graphs, and two RB siRNAs significantly reduced the level of RB compared with the negative control (Fig. 2a). SA-β-Gal staining was next used to assess corneal endothelium senescence, and our results showed that the percentage of SA-β-Gal-positive RCECs was reduced upon RB knockdown (Fig. 2b and c). A RT-PCR assay was used to measure the expression level of the senescence-related gene p21. Our results showed that the expression of the senescence-related gene p21 was reduced at the transcription level in the sample with siRb (Fig. 2d), and the expression of 5 secreted factors (CCL2, IL-1α, IL-1, IL-6, and IL-8) was also significantly decreased (Fig. 2e). These results suggested that siRb inhibits RCEC senescence.

3.3 Effect of siRB on cell proliferation and functional phenotype of RCECs

First, we wanted to know whether reducing RB might result in cell proliferation in RCECs. Cell cycle results proved that the numbers of cells at the G2/M phase and at the S phase were significantly increased following knockdown of RB and 72 subsequent hours of culture (Fig. 3a). The potential effect of RB on wound restoration was evaluated by a migration assay, and the results showed that the wound restoration capacity of cells following RB knockdown was also not significantly different from that of NC (Fig. 3b and c). N-Cadherin and ZO-1, which play roles in pump and barrier functions, were assessed by immunofluorescence, and their expression was the same as that of the control (Fig. 3d). These results suggest that siRb did not influence cell proliferation or the functional phenotype of RCECs.

3.4 Rb may induce RCEC senescence by inhibiting E2F2 activation

RB promotes cellular senescence; thus, we tested whether knocking down RB was associated with the activation of E2F. The tumor suppressor RB gene product pRB triggers senescent growth arrest when inactivated. When inactivated, E2F is tightly bound to pRB and prevents activation of E2F and unregulated DNA synthesis. RT-PCR experiments were employed to measure the expression of E2F2 and

E2F4, and the results showed that knocking down RB upregulated E2F2 (Fig. 4a), suggesting that RB may promote the senescence of corneal endothelial cells by inhibiting the activation of E2F2.

4. Discussion

As experimental and clinical evidence has shown, Fuchs endothelial dystrophy (FED), chronic corneal allograft failure, and cell density decreases when cells are cultured; in other words, cells exhibit characteristics of senescence.[9, 12, 30] The characteristics of cellular senescence include a senescence-like morphology with enlarged cell bodies, an irregular shape, and an increased proportion of multinucleated cells. Our team tried to expand HCECs in vitro under standard culture conditions, but it was difficult to maintain the morphology and function after 2–3 passages. After that, our research group attempted to apply conditioned medium to promote cell proliferation and maintain morphology in RCECs. Treatment with conditioned medium resulted in an increase in cell proliferation, and cells exhibited hexagonal morphology; further, cell pump function evaluation revealed complete functionality. However, there was no significant improvement in human corneal endothelial cells. The greatest challenge is still cells losing density after several cell passages because of cellular senescence. However, the underlying mechanisms of senescence in CE cells are largely unclear. Cellular senescence is identified by positive SA-β-gal staining, high expression of cyclin-dependent kinase inhibitors such as $p16^{INK4a}$, $p21^{CIP1}$, $p27^{KIP1}$ or $p53^{14}$, enlarged cell size, and the acquisition of an associated secretory phenotype (SASP).

Cellular senescence can be stimulated by inducers, such as H_2O_2 and TGF-β.[31, 32] It can also be produced by several days of culture.[9] In our study, rabbit corneal endothelial cells were cultured in standard medium for 5 to 7 days and then were passaged at a 1:3 ratio using a trypsin-EDTA solution. As the passage number increased, RCECs gradually became larger, the cell morphology gradually shifted to an irregular shape from a round shape, the cell density declined, and the percentage of SA-β-gal-positive cells was upregulated as the cell passage increased. Furthermore, compared with P1, the expression of 7 cytokines and chemokines (CCL2, CXCL10, TNF, IL-1α, IL-1β, IL-6, and IL-8) was significantly upregulated in P3. Moreover, the expression of the senescence-related gene p21 also increased at the mRNA level. We used siRNAs to knock down RB, and our results showed weak positive SA-β-gal staining and low expression of $p21^{CIP1}$ following RB knockdown. We also found that cell size was smaller than that of the NC cells. Cellular senescence is a special, irreversible state of cycle arrest, and knocking down RB significantly increased the cell cycle but had no influence on the migratory ability or functional phenotype.

In addition, the RB gene product is pRB, which is a tumor suppressor gene.[26] When inactivated, E2F is tightly bound to pRB, which prevents activation of E2F, disrupts regulation of DNA synthesis, and triggers senescent growth arrest. [1] Therefore, we assessed the expression levels of E2F2 and E2F4 after knocking down RB and found that E2F2 expression was significantly upregulated at the mRNA level, which explained the effect of Rb knockdown on the cell cycle and cell senescence.

Certainly, there are many limitations in our research. First, because human corneal endothelial cells are difficult to obtain and culture, all the studies in this paper were on rabbit corneal endothelial cells. In addition, as the proteins studied in our study used rabbit antigens, it was difficult to use antibodies to assess the corresponding protein levels with WB; therefore, the results in this paper are mostly limited to the mRNA level. Furthermore, the mechanism of the effect of Rb on cell senescence still needs further study, but our work provides a theoretical basis of an anti-senescent treatment for corneal endothelial cells.

5. Conclusions

We demonstrated that as the cells increased in passage number, the number of senescent RCECs, the levels of the senescence-related genes p21, and the levels of senescence-associated secretory factors increased. Retinoblastoma (Rb) promoted the senescence of corneal endothelial cells by inhibiting the activation of E2F2.

6. Abbreviations

CECs	Corneal endothelial cells
Rb	Retinoblastoma
FED	Fuchs endothelial dystrophy
CCAD	chronic corneal allograft dysfunction

7. Declarations

7.1 Ethics approval and consent to participate

Rabbits were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experiments were approved by the university's Animal Care and Use Committee.

7.2 Consent for publication

Not applicable.

7.3 Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

7.4 Competing interests

None of the authors have conflicts of interests to declare.

7.5 Funding

This work was mainly supported by grants from the National Natural Science Foundation of China (No. 81560158 to HGF), the Youth Science Foundation of Jiangxi Province (No. 20161BAB215188 to HGF), and a Talent team scheme - academic and technical leaders funding scheme for major disciplines (20172BCB22029 to HGF). The funders had no role in the study design, data collection, analysis and interpretation, or in writing the manuscript.

7.6 Authors' Contributions

AL and LB performed the experiments described. AL analyzed the data and wrote the manuscript. SJ, LL, WW, and YY assisted with the animal experiments. GF, YS, conceived of the study. All authors assessed and approved the final manuscript.

7.7 Acknowledgments

Not applicable.

7.8 Authors' information

Alan Jiang, Email:1426734362@qq.com

Longbing Mao, Email:602320556@qq.com

Sujuan Duan, Email:869616650@qq.com

Yanyan Zhang, Email:1322391287@qq.com

Xing Liu, Email:987902781@qq.com

Wenwen Cui, Email:www.cuiwenwen@qq.com

Yi Sang, Email:cqsy10@sina.com

Guofu Huang, Email:hgf2222@sina.com

7.9 Footnotes

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. The first tow authors contributed equally to the work.

References

1. Joyce, N.C., *Proliferative capacity of the corneal endothelium.* Prog Retin Eye Res, 2003. **22**(3): p. 359-89.
2. Sheerin, A.N., et al., *Characterization of cellular senescence mechanisms in human corneal endothelial cells.* Aging Cell, 2012. **11**(2): p. 234-40.
3. Bonanno, J.A., *Molecular mechanisms underlying the corneal endothelial pump.* Exp Eye Res, 2012. **95**(1): p. 2-7.
4. Joyce, N.C., *Cell cycle status in human corneal endothelium.* Exp Eye Res, 2005. **81**(6): p. 629-38.
5. Hoffer, K.J., *Corneal Endothelial Cell Density in Children: Normative Data From Birth to 5 Years Old.* Am J Ophthalmol, 2017. **178**: p. 186.
6. Morishige, N. and K.H. Sonoda, *Bullous keratopathy as a progressive disease: evidence from clinical and laboratory imaging studies.* Cornea, 2013. **32 Suppl 1**: p. S77-83.
7. Song, Q., et al., *Directed differentiation of human embryonic stem cells to corneal endothelial cell-like cells: A transcriptomic analysis.* Exp Eye Res, 2016. **151**: p. 107-14.
8. He, S. and N.E. Sharpless, *Senescence in Health and Disease.* Cell, 2017. **169**(6): p. 1000-1011.
9. Wei, X., et al., *Kojic acid inhibits senescence of human corneal endothelial cells via NF-kappaB and p21 signaling pathways.* Exp Eye Res, 2019. **180**: p. 174-183.
10. Jurkunas, U.V., *Fuchs Endothelial Corneal Dystrophy Through the Prism of Oxidative Stress.* Cornea, 2018. **37 Suppl 1**: p. S50-s54.
11. Matthaei, M., et al., *Endothelial Cdkn1a (p21) overexpression and accelerated senescence in a mouse model of Fuchs endothelial corneal dystrophy.* Invest Ophthalmol Vis Sci, 2012. **53**(10): p. 6718-27.
12. Li, Z., et al., *TGF-beta induces corneal endothelial senescence via increase of mitochondrial reactive oxygen species in chronic corneal allograft failure.* Aging (Albany NY), 2018. **10**(11): p. 3474-3485.
13. Lass, J.H., et al., *Donor age and corneal endothelial cell loss 5 years after successful corneal transplantation. Specular microscopy ancillary study results.* Ophthalmology, 2008. **115**(4): p. 627-632.e8.
14. Okumura, N., et al., *ROCK inhibitor converts corneal endothelial cells into a phenotype capable of regenerating in vivo endothelial tissue.* Am J Pathol, 2012. **181**(1): p. 268-77.
15. Koizumi, N., et al., *Cultivated corneal endothelial cell sheet transplantation in a primate model.* Invest Ophthalmol Vis Sci, 2007. **48**(10): p. 4519-26.
16. Sun, P., et al., *Promoting the expansion and function of human corneal endothelial cells with an orbital adipose-derived stem cell-conditioned medium.* Stem Cell Res Ther, 2017. **8**(1): p. 287.
17. Ali, M., et al., *Generation and Proteome Profiling of PBMC-Originated, iPSC-Derived Corneal Endothelial Cells.* Invest Ophthalmol Vis Sci, 2018. **59**(6): p. 2437-2444.

18. Chen, X., et al., *Directed Differentiation of Human Corneal Endothelial Cells From Human Embryonic Stem Cells by Using Cell-Conditioned Culture Media.* Invest Ophthalmol Vis Sci, 2018. **59**(7): p. 3028-3036.
19. Nakahara, M., et al., *Effect of a p38 Mitogen-Activated Protein Kinase Inhibitor on Corneal Endothelial Cell Proliferation.* Invest Ophthalmol Vis Sci, 2018. **59**(10): p. 4218-4227.
20. Childs, B.G., et al., *Cellular senescence in aging and age-related disease: from mechanisms to therapy.* Nat Med, 2015. **21**(12): p. 1424-35.
21. Campisi, J., *Aging, cellular senescence, and cancer.* Annu Rev Physiol, 2013. **75**: p. 685-705.
22. Sharpless, N.E. and C.J. Sherr, *Forging a signature of in vivo senescence.* Nat Rev Cancer, 2015. **15**(7): p. 397-408.
23. Nardella, C., et al., *Pro-senescence therapy for cancer treatment.* Nat Rev Cancer, 2011. **11**(7): p. 503-11.
24. Sedelnikova, O.A., et al., *Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks.* Nat Cell Biol, 2004. **6**(2): p. 168-70.
25. van Deursen, J.M., *The role of senescent cells in ageing.* Nature, 2014. **509**(7501): p. 439-46.
26. Shamma, A., et al., *Rb Regulates DNA damage response and cellular senescence through E2F-dependent suppression of N-ras isoprenylation.* Cancer Cell, 2009. **15**(4): p. 255-69.
27. Fisher, R.P. and D.O. Morgan, *A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase.* Cell, 1994. **78**(4): p. 713-24.
28. Kato, J., et al., *Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4.* Genes Dev, 1993. **7**(3): p. 331-42.
29. Duan, S., et al., *Histone deacetylase inhibitor, AR-42, exerts antitumor effects by inducing apoptosis and cell cycle arrest in Y79 cells.* J Cell Physiol, 2019. **234**(12): p. 22411-22423.
30. Hongo, A., et al., *The Effect of a p38 Mitogen-Activated Protein Kinase Inhibitor on Cellular Senescence of Cultivated Human Corneal Endothelial Cells.* Invest Ophthalmol Vis Sci, 2017. **58**(9): p. 3325-3334.
31. Li, Z.Y., et al., *TGF-beta and NF-kappaB signaling pathway crosstalk potentiates corneal epithelial senescence through an RNA stress response.* Aging (Albany NY), 2016. **8**(10): p. 2337-2354.
32. Zhao, X., et al., *Oxidative stress and premature senescence in corneal endothelium following penetrating keratoplasty in an animal model.* BMC Ophthalmol, 2016. **16**: p. 16.

Figures

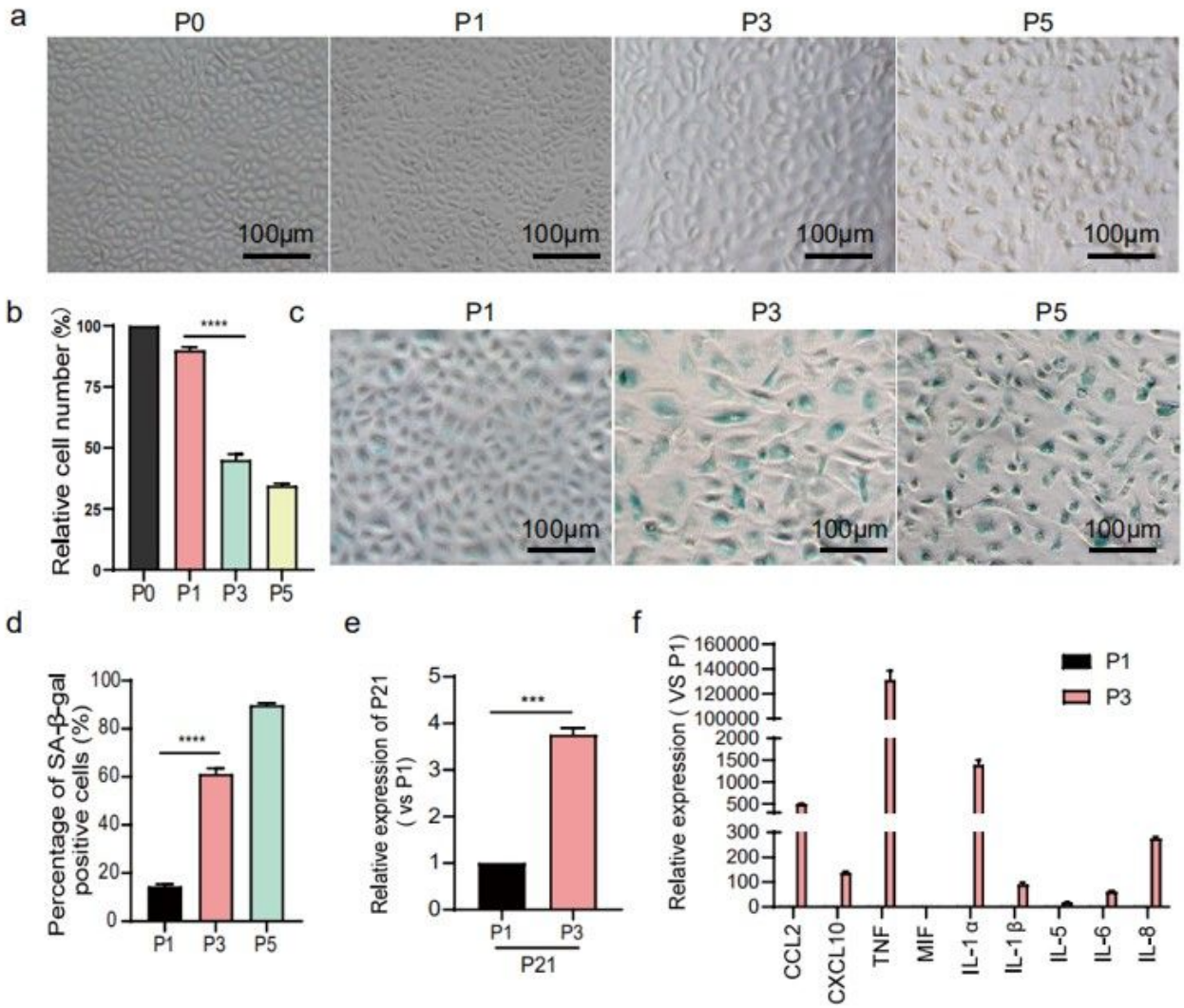

Figure 1

Corneal endothelial cell senescence and senescence-associated secretion of factors after the passaging the cells. (A and B) Phase-contrast images of RCEC cultures. RCECs were passaged 0 to 5 times, and microscopy images indicated the shape and cell size. (C and D) SA-β-Gal staining of CEC cultures. The senescing cells were assessed for SA-β-Gal activity. The graph represents the number of cells associated with senescing cells. (E and F) RT-PCR measured the level of the senescence-related gene p21 and of the SASP factors. Data are represented as the average values of triplicate analyses of three independent experiments. (***p<0.001, and ****p<0.0001; n=3).

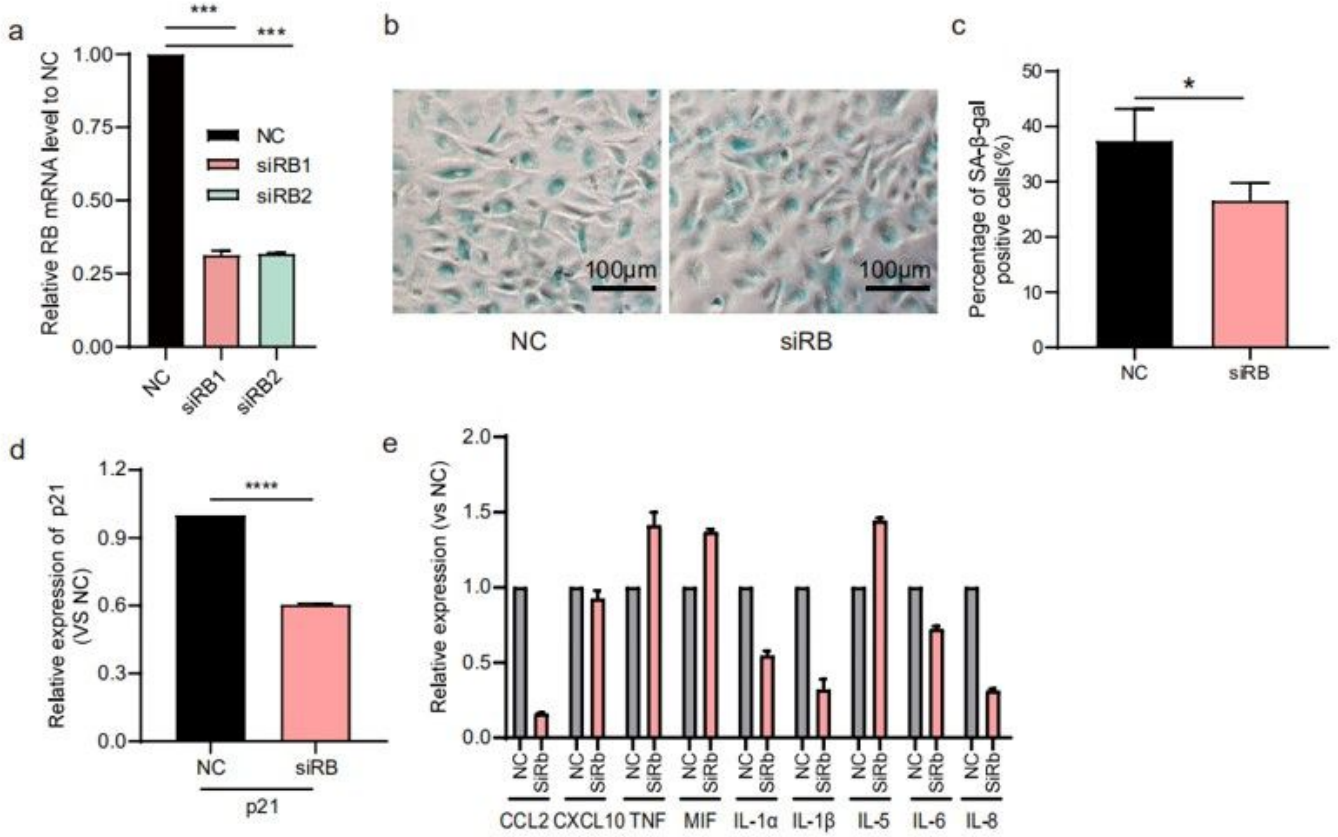

Figure 2

Knocking down Rb suppresses senescent phenotypes. (A) RT-PCR was used to assess the interference efficiency of siRb. (B and C) SA-β-Gal staining of RCECs treated with siRb. The senescing cells were assessed for SA-β-Gal activity. The graph represents the number of identified senescent cells. (D and E) qPCR measured the level of p21 and the SASP factors. (*P < 0.05, ***P < 0.001, and ****P < 0.0001; n =3).

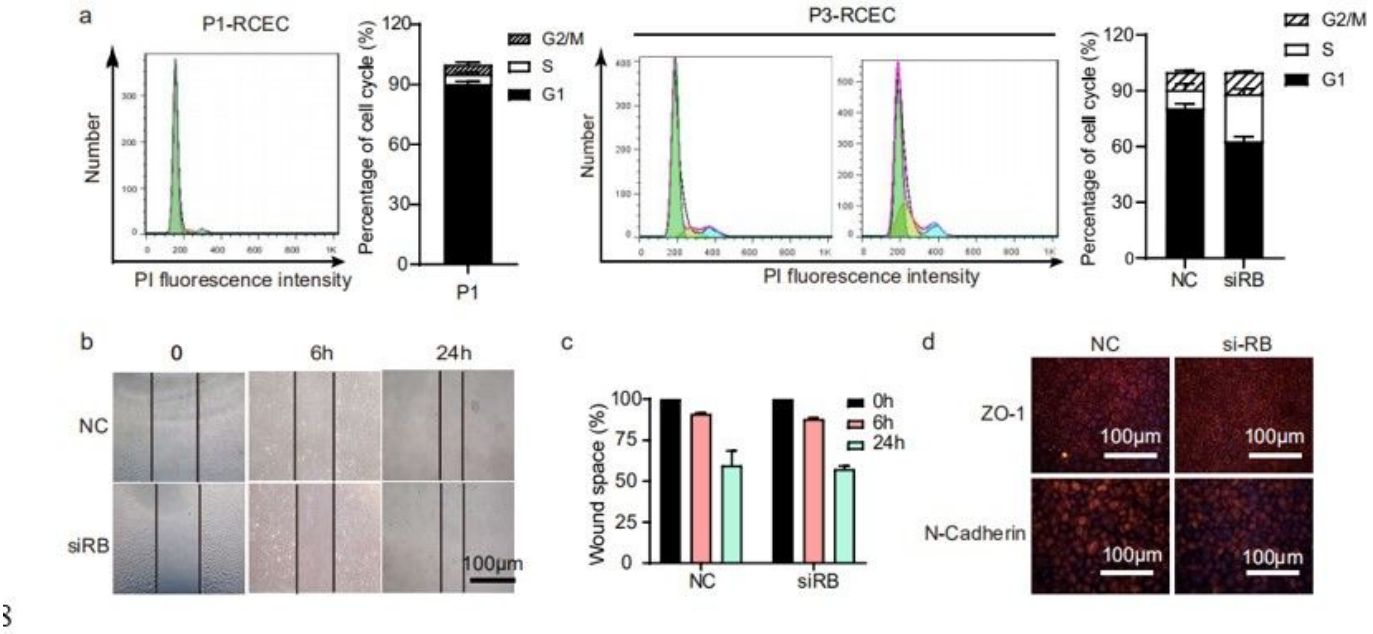

Figure 3

Knocking down Rb did not influence the cell cycle, migratory ability or functional phenotype. (A) The cell cycle was evaluated using flow cytometry. (B and C) The migratory ability of RCECs was evaluated by wound healing assays. The graph represents the percentage of remaining wound area. (D) The function-related markers ZO-1 and N-cadherin were assessed by immunostaining.

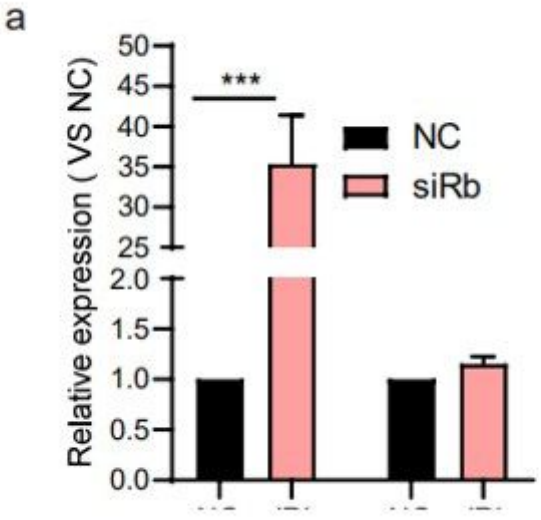

Figure 4

Rb may induce RCEC senescence by inhibiting E2F2 activation. (A) RT -PCR was used to measure the level of E2F. (***P < 0.001; n =3).