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Brief research report: Transcriptional blockade of angiotensin converting enzyme 2 modelled in human retinal pigment epithelial cells

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As a key host protein involved in cellular infection by the severe acute respiratory syndrome coronavirus (SARS-CoV-2), angiotensin converting enzyme (ACE)2 is an ideal target for antiviral drugs. Manipulation of transcription provides opportunity for graduated blockade that preserves physiological functions. We sought to develop a model system for evaluating manipulation of ACE2 gene transcription using human retinal pigment epithelium. Retinal pigment epithelial cell isolates were prepared from human posterior eyecups (n = 11 individual isolates). The cells expressed ACE2 transcript and protein, and expression was not induced by hypoxia mimetic dimethylxaloylglycine, or inflammatory cytokine IL-1 β . ACE2 gene transcription factors were predicted *in silico* and cross-referenced with the human retinal pigment epithelial cell transcriptome, and five candidate transcription factors were identified: ETS proto-oncogene 1 transcription factor (ETS1), nuclear factor I C (NFIC), nuclear receptor subfamily 2 group C member 1 (NR2C1), TEA domain transcription factor 1 (TEAD1), and zinc finger protein 384 (ZNF384). The candidates were individually targeted in cells by transfection with small interfering (si)RNA. Knockdowns reduced mean cellular expression of all the transcription factors in comparison to expression in cells transfected with control non-targeted siRNA. Mean cellular ACE2 transcript was reduced under the condition of NR2C1 knockdown, but not for ETS1, NFIC, TEAD1, and ZNF384 knockdowns. Our findings build on previous work demonstrating the potential for drugging gene transcription. Importantly, we show the value of human retinal pigment epithelium as a system for evaluating ACE2 transcriptional blockade, a possible approach for treating SARS-CoV-2 infection. Brief Research Report.

KEYWORDS

transcription, ACE2, NR2C1, retinal pigment epithelium, drugging

1 Introduction

Measurements of excess mortality for over 70 countries and territories indicate that approximately 18.2 million people died in 2020 and 2021 as a result of the coronavirus disease 2019 (COVID-19) pandemic (COVID-19 Excess Mortality Collaborators, 2022). The pandemic has not yet ended, with new waves of infection continuing to arise (Giovanetti et al., 2023). A range of vaccines have been developed against infection with the causative virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Eroglu et al., 2021; Fiolet et al., 2022; Li et al., 2022). These vaccines are effective in inducing an immune response and lowering the risk of infection (Tregoning et al., 2021). For additional protection of individuals at risk of severe disease and those people who are not vaccinated, anti-viral drugs that target the replication of SARS-CoV-2 have been introduced into clinical care (Hui and Zumla, 2022; Lai et al., 2022; Nazir et al., 2024). In evaluating these major advances, a group of experts have called for diversification in anti-viral drug development, suggesting new therapeutics should include drugs that target host molecules involved in viral replication (Edwards et al., 2022).

Angiotensin converting enzyme (ACE)2 is a host molecule that is essential for the replicative lifecycle of SARS-CoV-2 (Scialo et al., 2020; Hu et al., 2021). This carboxypeptidase is a key component of the renin-angiotensin system that controls the body's fluid and electrolyte balance, as well as the blood pressure (Clarke and Turner, 2012; Verano-Braga et al., 2020). Critically, ACE2 converts angiotensin (Ang) II to Ang (1-7), and Ang I to Ang (1-9) (Clarke and Turner, 2012; Verano-Braga et al., 2020). The molecule has additional substrates, and beyond the enzymatic activity, it has important roles in metabolism and inflammation (Aleksova et al., 2021). The spike (S) protein of SARS-CoV-2 recognizes membrane-bound ACE2 as the first step in viral infection of a human cell (Hu et al., 2021). Because of its specific interaction with the S protein, ACE2 is an appealing potential drug target for the treatment of COVID-19 (Mostafa-Hedeab, 2020; Singh et al., 2022; Suvarnapathaki et al., 2022; Vitiello and Ferrara, 2022). However, given the multiple important physiological roles of ACE2, there are valid concerns about inhibiting this molecule, including the potential to aggravate COVID-19 by compromising cardiovascular function (Cousin et al., 2021; Momtazi-Borojeni et al., 2021; Zhu et al., 2021).

Manipulating transcription provides the opportunity for graduated blockade of a target molecule (Ashander et al., 2016). As demonstration of this concept, we achieved a partial reduction in the level of intercellular adhesion molecule (ICAM)-1 on human endothelial cells by silencing the transcription factors, C2 calcium dependent domain containing 4B (C2CD4B) and interferon regulatory factor 1 (IRF1) (Haydinger et al., 2023; Ma et al., 2023). In the study described in this Brief Research Report, we developed a model system for testing the manipulation of ACE2 gene transcription. We predicted *in silico* transcription factors that might control ACE2 gene expression, and then evaluated the impact of small interfering (si)RNA-mediated knockdown of the candidates in human epithelial cells. We used retinal pigment epithelial cells obtained from human cadaveric donor eyes for the system for several reasons. Firstly, there are robust methods for the isolation and characterization of this cell population (Blenkinsop et al., 2013;

Smith et al., 2020; Markert et al., 2022). Secondly, these cells are highly receptive to transfection (Feng et al., 2022; Hu X. et al., 2022b; Mattern et al., 2022). Thirdly, as we confirm in this work, human retinal pigment epithelial cells express ACE2 (Yuan et al., 2020; Eriksen et al., 2021; Albertos-Arranz et al., 2023).

2 Materials and methods

2.1 Isolation of human retinal pigment epithelial cells

Retinal pigment epithelial cell isolates were prepared from paired human cadaveric donor eyes obtained from the Eye Bank of South Australia (Adelaide, Australia). The isolation method has been detailed in previous publications (Ashander et al., 2019; Lie et al., 2019; Ashander et al., 2022), and yields cells that express cytokeratin-8, retinal pigment epithelium-specific protein 65, cellular retinaldehyde-binding protein, and zonula occludens 1, and not the mesenchymal differentiation marker, α -smooth muscle actin (Smith et al., 2020).

In brief, the retinal pigment epithelium-choroid was dissected from posterior eyecups and incubated with 0.5 mg/mL collagenase IA and 0.5 mg/mL collagenase IV (Merck-Sigma Aldrich, St Louis, MO) in Hanks' Balanced Salt Solution (HBSS) for 30 min at 37°C and 5% CO₂ in air, and subsequently rinsed with Dulbecco's phosphate buffered saline (DPBS) with 2% fetal bovine serum (FBS, Bovogen Biologicals, Keilor East, Australia). Sheets of retinal pigment epithelium were gently scraped off Bruch's membrane with a spatula at the dissecting microscope and suspended in fresh DPBS with 2% FBS. Suspended cell sheets were pelleted by centrifugation and resuspended in DPBS with 2% FBS, and subsequently layered onto 10% sucrose solution in DPBS with 2% FBS, and centrifuged to collect the cell sheets. Sheets were resuspended in 50% Minimum Essential Medium Eagle alpha modification (with sodium bicarbonate) (MEM), 25% Dulbecco's Modified Eagles Medium (DMEM) and 25% F-12 with 1x N1 Medium Supplement, 1x Non-Essential Amino Acids Solution, 1x GlutaMAX Supplement, 0.25 mg/mL taurine, 0.02 μ g/mL hydrocortisone, 0.013 ng/mL 3,3',5-triiodo-L-thyronine sodium, 100 U/mL Penicillin-Streptomycin (all obtained from Merck-Sigma Aldrich or Thermo Fisher Scientific-Gibco, Grand Island, NY), and 2% FBS. Sheets were distributed in a dish to 50% covered, and cultured at 37°C and 5% CO₂ in air until confluent. In some experiments, cells were treated with 1 mM dimethylxaloylglycine (DMOG) (Merck-Sigma Aldrich) or 10 ng/mL human recombinant interleukin (IL)-1 β (R&D Systems, Minneapolis, MN).

2.2 Immunocytofluorescence

Primary human retinal pigment epithelial cells were plated in glass bottom chamber slides (Ibidi GmBH, Grafelfing, Germany) and grown to confluence at 37°C and 5% CO₂ in air. Medium was removed, and monolayers were washed twice in DPBS prior to fixation for 10 min in 4% paraformaldehyde at room temperature. All subsequent steps were carried out in room temperature unless

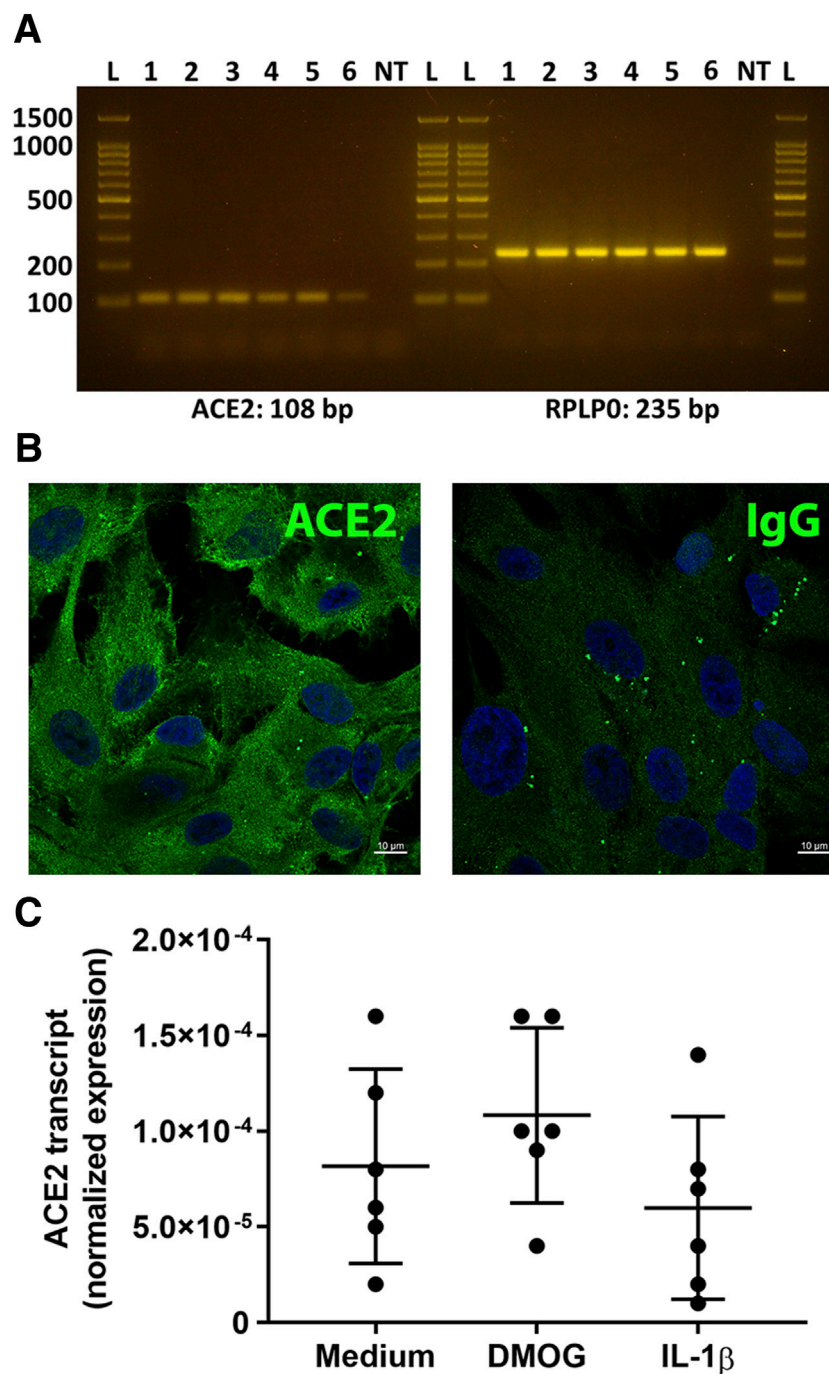


FIGURE 1

Expression of ACE2 by primary human retinal pigment epithelial cells. (A) Gel images show ACE2 and reference gene RPLP0 amplicons. Lanes: L = DNA ladder (indicating 100, 200, 500, 1,000, 1,500 base pair markers); 1-6 = primary human retinal pigment epithelial cell isolates from six individual donors; NT = no cDNA template control. Expected product size is stated in base pairs (bp) below gel image. (B) Fluorescence photomicrographs of human retinal pigment epithelial cells immunolabelled to detect the presence of ACE2, with negative control labelled with species-matched immunoglobulin (IgG). Alexa Fluor 488 (green) with DAPI nuclear counterstain (blue). Scale bar represents 10 microns. Representative of results obtained for three individual cell isolates. (C) Graph showing normalized expression of ACE2 transcripts in 6 cell isolates treated with dimethylxaloylglycine (DMOG) or interleukin (IL)-1 β , calculated relative to PPIA and YWHAZ. Circles represent individual cell isolates; crossbars indicate mean; and error bars indicate standard deviation. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test applied to compare individual groups: there were no significant differences between control and treatment groups.

noted. After an additional three washes in non-Dulbecco's phosphate buffered saline (PBS), monolayers were blocked for 1 h in PBS containing 5% normal rabbit serum (Vector Labs, Burlingame, MA), and then incubated for 1 h with 7.5 μ g/mL polyclonal goat

anti-human ACE2 antibody (R&D Systems, Waltham, MA: catalogue number AF933) or purified goat immunoglobulin (IgG) (Vector Labs) in PBS containing 5% normal rabbit serum and 0.05% saponin. Primary antibody incubation was continued overnight at 4°C, after

TABLE 1 Sequences and locations of putative transcription factor binding sites within the regulatory region of the human *ACE2* gene. Gene sequences were obtained from the JASPAR 2020 database (Fornes et al., 2020).

Transcription factor	Predicted sequence	Location		
		Start	End	Strand
ETS1	CATCCT	121	126	+
	CATCCT	2,818	2,823	-
	TTCCT	428	433	+
	TTCCT	747	752	+
	TTCCT	1,055	1,060	+
	TTCCT	1,545	1,550	+
	TTCCT	1,582	1,587	-
	TTCCT	2,013	2,018	-
	TTCCT	2,672	2,677	-
	TTCCT	2,884	2,889	+
	TTCCG	944	949	+
	TTCCG	2,646	2,651	+
NFIC	CTGGCA	1,770	1,775	+
	TTGGCT	733	738	+
	TTGGCT	2,833	2,838	+
	TTGGCT	2,982	2,987	+
NR2C1	TAAGGTCAA	358	366	+
	CAAGGTCAG	2,189	2,197	+
	CAAGGTCAC	1,930	1,938	-
	CGAGGTCAG	101	109	+
TEAD1	CACATTCTAA	16	25	+
	CACATTCCAG	1,060	1,069	-
	TACATTCCCTT	487	496	+
	CCACATTCCAGGA	1,058	1,070	-
ZNF384	CTCAAAAAAAAA	317	328	+
	CTCAAAAAAAAA	2,404	2,415	+
	AAAAAAAAAAAC	2,418	2,429	+
	AAAAAAAAAAAT	322	333	+
	AAAAAAAAAAAT	1,098	1,109	-
	TTTAAAAAAAAATA	1,172	1,183	-
	GGTAAAAAAAAA	1,102	1,113	-
	CAAAAAAAAAA	319	330	+
	CAAAAAAAAAA	2,406	2,417	+
	GTAAAAAAAAA	1,101	1,112	-
	TCTCAAAAAAAAA	316	327	+
	TCTCAAAAAAAAA	2,403	2,414	+
	TCAAAAAAAAAA	318	329	+

(Continued on following page)

TABLE 1 (Continued) Sequences and locations of putative transcription factor binding sites within the regulatory region of the human *ACE2* gene. Gene sequences were obtained from the JASPAR 2020 database (Fornes et al., 2020).

Transcription factor	Predicted sequence	Location		
		Start	End	Strand
	TCAAAAAAAAAA	2,405	2,416	+
	AAAAAAAAAAAA	320	331	+
	AAAAAAAAAAAA	321	332	+
	AAAAAAAAAAAA	1,099	1,110	-
	AAAAAAAAAAAA	2,407	2,418	+
	AAAAAAAAAAAA	2,408	2,419	+
	AAAAAAAAAAAA	2,409	2,420	+
	AAAAAAAAAAAA	2,410	2,421	+
	AAAAAAAAAAAA	2,411	2,422	+
	AAAAAAAAAAAA	2,412	2,423	+
	AAAAAAAAAAAA	2,413	2,424	+
	AAAAAAAAAAAA	2,414	2,425	+
	AAAAAAAAAAAA	2,415	2,426	+
	AAAAAAAAAAAA	2,416	2,427	+
	AAAAAAAAAAAA	2,417	2,428	+
	TAAAAAAAAAAA	1,100	1,111	-

Abbreviations: ACE2, angiotensin converting enzyme 2; ETS1, ETS, proto-oncogene 1, transcription factor; NFIC, nuclear factor 1 C; NR2C1, nuclear receptor subfamily 2 group C member 1; TEAD1 = TEA, domain transcription factor 1; ZNF384 = zinc finger protein 384.

which monolayers were washed 3 times in PBS and incubated for 45 min with 2.5 µg/mL Alexa Fluor 488-conjugated rabbit anti-goat IgG (Thermo Fisher Scientific-Molecular Probes, Eugene, OR) in PBS containing 5% normal rabbit serum and 0.05% saponin. Monolayers were washed again in PBS and fixed for 10 min in 4% paraformaldehyde prior to counterstaining for 5 min with 300 nM 4',6-diamidino-2-phenylindole (DAPI, Merck-Sigma Aldrich). After additional washes in PBS, stained monolayers were mounted in Fluoromount aqueous mounting medium (Merck-Sigma Aldrich) and imaged on the LSM 880 Fast Airyscan confocal microscope (Carl Zeiss AG, Oberkochen, Germany) under a 63x/1.4 objective with oil immersion. Images were processed using ZEN microscopy software v3.2 (Carl Zeiss AG).

2.3 RNA extraction and reverse transcription

Retinal pigment epithelial cell monolayers were treated with Buffer RLT (Qiagen, Hilden, Germany) containing 1:100 2-mercaptoethanol and subsequently frozen at -80°C. After thawing, RNA was extracted using the RNeasy Mini Kit (Qiagen) with optional on-column DNase I digestion, according to the manufacturer's protocol. Nucleic acid concentration was determined by spectrophotometry using the Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE). The reverse transcription (RT) was carried out using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Hercules, CA) with 250 ng RNA input

per reaction. Duplicate cDNA synthesis reactions were prepared for each sample and the resulting cDNA was pooled prior to the polymerase chain reaction (PCR).

2.4 Standard polymerase chain reaction

Standard PCR was carried out on the T100 Touch Thermocycler (Bio-Rad Laboratories). In addition to nuclease-free water and 1X PCR buffer, each reaction contained 0.4 mM dNTP mix, 1.5 mM or 5 mM (ETS1) MgCl₂, 0.25 µM or 0.8 µM (ETS1) each of forward and reverse primer, 1 unit of HotStarTaq Plus DNA polymerase (Qiagen), and 2 µL of neat cDNA template. Cycling conditions were as follows: 5-min pre-amplification hold at 95°C followed by 40 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 61°C (ETS1: 58°C), extension for 30 s at 72°C, and a post-amplification hold of 72°C for 5 min. The PCR product sizes were confirmed by 2% agarose gel electrophoresis. Ribosomal protein lateral stalk subunit P0 (RPLP0) was used as a reference gene.

2.5 Real-time quantitative polymerase chain reaction

To assess reduction of targeted transcripts by siRNA and the accompanying *ACE2* gene expression changes, real-time quantitative PCR was performed on a CFX Connect Real-Time PCR System (Bio-

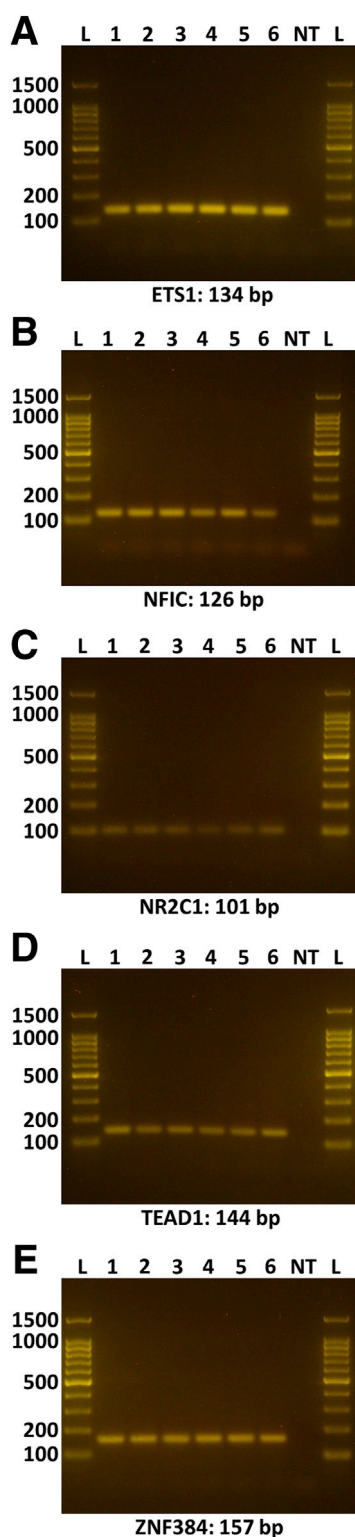


FIGURE 2
Expression of selected transcription factors in human retinal pigment epithelial cells. Gel images show amplicons representing the following transcription factors: (A) ETS1, (B) NFIC, (C) NR2C1, (D) TEAD1, (E) ZNF384. Lanes: L = DNA ladder (indicating 100, 200, 500, 1,000, 1,500 base pair markers); 1-6 = primary human retinal pigment epithelial cell isolates from six individual donors; NT = no cDNA template control. Expected product size is stated in base pairs (bp) below gel image.

Rad Laboratories). In addition to SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories) and nuclease-free water, each reaction contained 300 nM of the forward and the reverse primer, and 2 μ L of undiluted cDNA. Cycling conditions included a pre-amplification hold of 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 30 s, and fluorescence reading after a 1 s hold at 75°C. Melting curves from 70°C to 95°C were performed for each run to confirm a single peak was produced for all primer sets, and amplicon sizes were confirmed by agarose gel electrophoresis. Relative expression of transcripts of interest, normalized to stable reference genes (i.e., coefficient of variation less than 0.25) peptidylprolyl isomerase A (PPIA) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ), was determined by the Pfaffl method (Pfaffl, 2001). Primer efficiency ranged from 78.5% to 93.4% with the exception of YWHAZ, where efficiency was 106.1%, as determined by CFX Manager software v3.0 (Bio-Rad Laboratories) using standard curves generated by serial dilution of purified PCR products. All primer sequences and expected product sizes are shown in Supplementary Table S1. For primers designed in-house, the product was confirmed by sequencing.

2.6 Identification of transcription factor candidates

Human *ACE2* gene sequence was obtained from the National Center for Biotechnology Information (NCBI) Nucleotide database (NG_012575.1: *Homo sapiens* angiotensin I converting enzyme 2 (*ACE2*), RefSeqGene on chromosome X: https://www.ncbi.nlm.nih.gov/nuccore/NG_012575.1/). The regulatory sequence, defined as 3,000 bp of sequence immediately upstream of the *ACE2* gene transcription start site, was interrogated for potential transcription factor binding sites using JASPAR 2020, release 8: <https://jaspar2020.genereg.net>. JASPAR is an open-access platform of 'curated, non-redundant transcription factor-binding profiles stored as position frequency matrices for transcription factors across multiple species in six taxonomic groups' (Fornes et al., 2020). The computation task was performed on the Vertebrata taxonomic group section of the JASPAR platform, setting *H. sapiens* as the search species. The list of predicted binding sites was cross-referenced with the human retinal pigment epithelial cell transcriptome, which we previously generated by RNA-sequencing, achieving 40.3×10^6 mean paired reads aligning to the human genome (Smith et al., 2020), to identify transcription factors that might impact *ACE2* gene transcription in this cell population. The transcriptome is lodged in the NCBI Gene Expression Omnibus (Series GSE132091: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132091>). The transcription factor candidates were prioritized for investigation in this work on the basis of: normalized read count of at least 100; minimum of three predicted binding sites; and activator function established by report in the peer-reviewed literature.

2.7 RNA silencing

Primary human retinal pigment epithelial cells were grown to confluence in 12-well plates (growth area 3.8 cm²) at 37°C and 5%

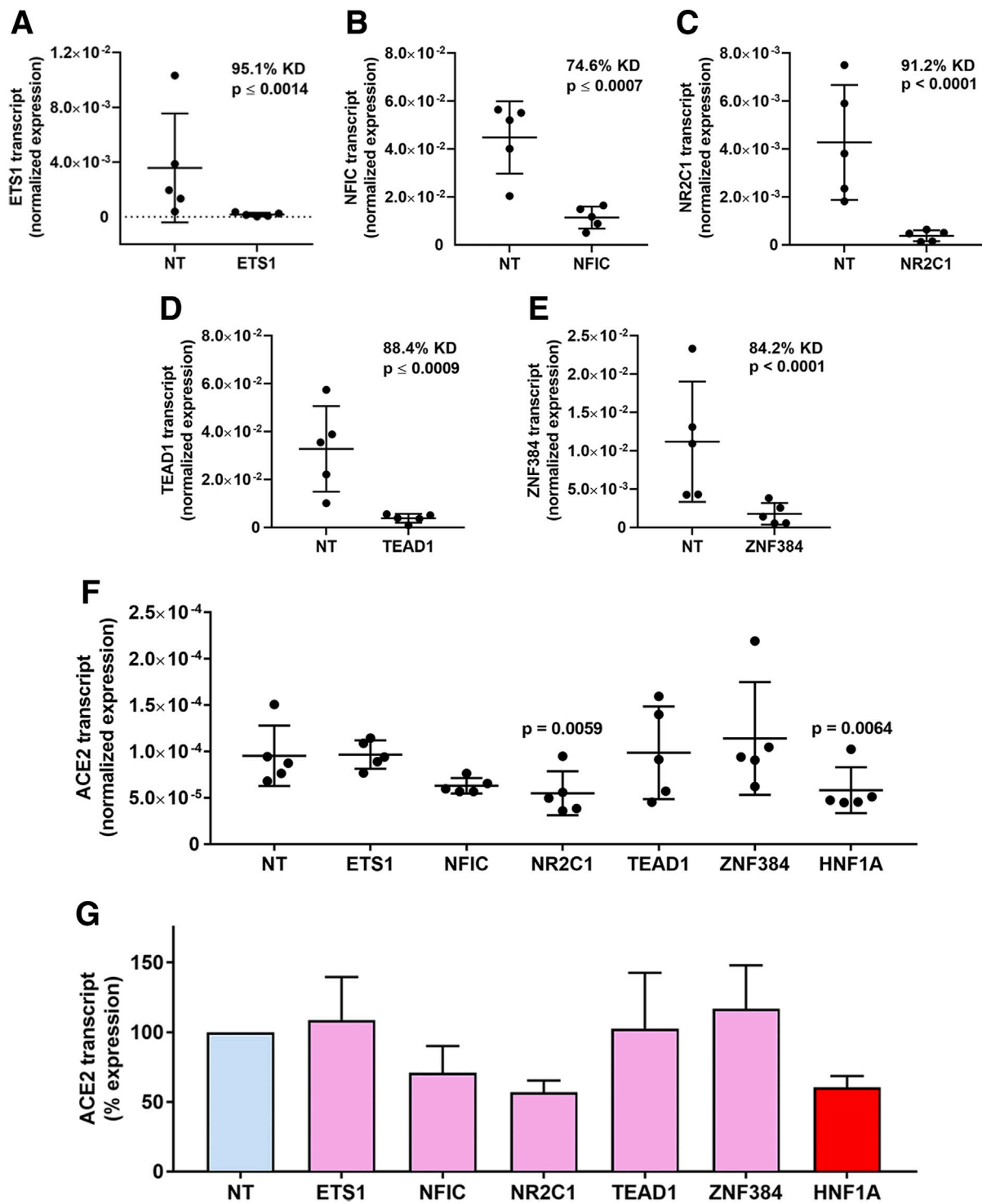


FIGURE 3 Transcription factor knockdown in human retinal pigment epithelial cells. (A–E) Graphs showing normalized expression of transcription factor transcripts, calculated relative to PPIA and YWHAZ, in human retinal pigment epithelial cell isolates treated with siRNA targeting (A) ETS1, (B) NFIC, (C) NR2C1, (D) TEAD1 and (E) ZNF384, or control non-targeted (NT) siRNA. Circles represent individual cell isolates ($n = 4$ monolayers per isolate); cross-bars indicate mean; and error bars indicate standard deviation. KD is knockdown effect expressed as percent. Data were analyzed by two-tailed paired Student's *t*-test. (F) Graph showing normalized expression of ACE2 transcript, calculated relative to PPIA and YWHAZ, across the same samples studied in (A–E). Circles represent individual cell isolates; crossbars indicate mean; and error bars indicate standard deviation. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test applied to compare individual groups. (G) Graph showing percentage changes in ACE2 transcript expression corresponding with normalized expression showed in (F). Bars indicate mean; and error bars indicate standard deviation.

CO₂ in air. Confluent monolayers were then treated with *Silencer Select* targeted siRNA (Supplementary Table S2) or the *Negative Control No.1* siRNA (Thermo Fisher Scientific-Ambion, Austin, TX). The siRNA were diluted in Opti-MEM I reduced serum medium (Thermo Fisher Scientific-Gibco), and combined with Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific-Ambion). Each well received 200 μ L of Opti-MEM I containing 12 pmol siRNA and 2 μ L of lipofectamine, for a final medium volume of 1.2 mL per well. Monolayers were incubated for 24 h at 37°C and 5% CO₂ in air, then siRNA-containing medium was replaced with 1 mL of fresh medium. Monolayers were incubated for a further 24–48 h, after which the medium was replaced with RLT lysis buffer in preparation for RNA extraction.

2.8 Data analysis

Data were analyzed using Prism versions 7.04 or 9.0.0 (GraphPad Software, La Jolla, CA). In comparisons of results for multiple human cell isolates, the two-tailed paired Student's *t*-test was employed for two conditions and the repeated measures one-way analysis of variance (ANOVA) with Geisser-Greenhouse correction, and with Dunnett's multiple comparisons test, was implemented for multiple conditions. Sample size determination and power analysis were not undertaken in this work which was planned as a feasibility study. Normality tests were not performed due to small sample numbers, following the recommendations stated in the Prism Statistics Guide. A statistically significant difference was defined by a *p*-value of less than 0.05 for all tests.

2.9 Human research ethics

Use of human cadaveric donor eyes for this research was approved by the Southern Adelaide Clinical Human Research Ethics Committee (protocol number: 175.13).

3 Results

Primary human retinal pigment epithelial cell isolates were prepared from paired eyecups of four men and seven women cadaveric donors, whose ages at death ranged from 46 to 78 years (median = 62 years). Time from death to processing of the human eyecups ranged from 14 to 41 h (median = 23 h).

Expression of ACE2 by human retinal pigment epithelial cells was investigated using isolates prepared from eyecups of multiple donors. Standard RT-PCR detected the ACE2 amplicon in RNA extracts obtained from each of six individual cell isolates (Figure 1A). Using indirect immunocytofluorescence and confocal microscopy, ACE2 protein was detected on monolayers of three different retinal pigment epithelial cell isolates; control monolayers immunolabelled with primary antibody directed against an irrelevant antigen showed no positive staining (Figure 1B). The real-time quantitative PCR showed over 3-fold variation in the level of transcript expression across cell isolates from six donors. ACE2 may be regulated by hypoxia and inflammatory cytokines in some settings (Clarke and Turner, 2012; Cousin et al., 2021).

However, 24-h treatments with the hypoxia mimetic DMOG, or the inflammatory cytokine IL-1 β , in standard concentrations, did not alter expression of ACE2 transcript in human retinal pigment epithelial cells (*p* > 0.05) (Figure 1C).

A search of 3,000 bp of sequence immediately upstream of the human ACE2 gene transcription start site on the JASPAR 2020 platform (Fornes et al., 2020) predicted a total of 255 potential cis-regulatory elements, which equated to binding sites for 93 different transcription factors (Supplementary Table S3). Cross-referencing this list with a human retinal pigment epithelial cell transcriptome, generated by RNA-sequencing (Smith et al., 2017), and prioritizing candidates on the basis of normalized read count, minimum predicted binding sites, and activator function, yielded five transcription factors: ETS proto-oncogene 1, transcription factor (ETS1, 12 cis-regulatory motifs), nuclear factor I C (NFIC, 4 cis-regulatory motifs), nuclear receptor subfamily 2 group C member 1 (NR2C1, 4 cis-regulatory motifs), TEA domain transcription factor 1 (TEAD1, 4 cis-regulatory motifs), and zinc finger protein 384 (ZNF384, 29 cis-regulatory motifs) (Table 1). Standard RT-PCR confirmed the expression of each of these transcription factors across human retinal pigment epithelial cell isolates from six donors (Figures 2A–E).

Individual targeted knockdowns for the candidate transcription factors, plus positive control hepatocyte nuclear factor 1 alpha (HNF1 α) (Pedersen et al., 2013), were performed in human retinal pigment epithelial cell isolates from five human donors using siRNA. This manipulation achieved highly significantly reduced mean expression across all the transcription factors (*p* \leq 0.0014), averaging 74.6%–95.1% in comparison to expression in cells transfected with control non-targeted siRNA (Figures 3A–E). Levels of the ACE2 transcript were determined by real-time quantitative RT-PCR in these same transfected cell isolates. This demonstrated significantly reduced mean ACE2 expression under the condition of NR2C1 knockdown (*p* = 0.0059), as well as positive control HNF1 α knock-down (*p* = 0.0064), and no significant changes in ACE2 expression for ETS1, NFIC, TEAD1 and ZNF384 knockdowns (*p* > 0.05) (Figure 3F). NR2C1 knockdown achieved an average 43.3% reduction in ACE2 transcript across the 5 cell isolates, similar to the 39.5% reduction for HNF1 α knock-down (Figure 3G).

4 Discussion

As the receptor for the SARS-CoV-2 S protein, ACE2 is an appealing target for new anti-viral drugs to treat COVID-19 (Mostafa-Hedeab, 2020; Singh et al., 2022; Suvarnapathaki et al., 2022; Vitiello and Ferrara, 2022). In this Brief Research Report, we have shown expression of ACE2 transcript and protein in the retinal pigment epithelium across multiple primary human cell isolates. By cross-referencing a list of *in silico*-predicted transcription factors involved in ACE2 gene transcription with the human retinal pigment epithelial cell transcriptome, we identified and then confirmed five candidate transcription factors. We observed that specific knockdown of NR2C1 significantly reduced the level of ACE2 transcript in these cells. Our findings build on previous work demonstrating the potential for drugging gene transcription (Ashander et al., 2016; Ma et al., 2023), and also demonstrate the value of human retinal pigment epithelial cells as a testing system.

The transcription factor, NR2C1, a member of the nuclear receptor superfamily subgroup 2C, was originally cloned from human testis and named testicular nuclear receptor 2 (TR2) (Lin et al., 2017). Subsequently the transcription factor was found in epithelia, including the retinal pigment epithelium (Baker et al., 2016). NR2C1 has been implicated in early neuroretinal development, participating in phenotypic development of photoreceptors and amacrine cells (Mollema et al., 2011; Olivares et al., 2017). While not known to be involved in retinal disease, limited work suggests possible roles for NR2C1 in oncogenesis (Safe et al., 2014; Sun et al., 2021; Liu et al., 2023).

Other investigators have predicted transcription factor targets for influencing *ACE2* gene expression in different model systems, typically drawing on *in silico* tools. One team used human lung and mouse tracheal RNA-sequencing datasets to identify binding sites for multiple transcription factors in the *ACE2* gene promoter, although they did not interrogate any candidates *in vitro* (Barker and Parkkila, 2020). An independent team (Liang et al., 2022) predicted a series of transcription factors from chromatin immunoprecipitation (ChIP)-on-chip and ChIP-sequencing datasets, and showed that the candidate signal transducer and activator of transcription (STAT)3 influenced expression of the *ACE2* gene in the immortalized human bronchial 16HBE cell line. Using repositied data generated from human and mouse non-specified epithelial cells, another group also predicted binding sites for STAT3 in the *ACE2* gene regulatory region, as well as for STAT1, interferon regulatory factor (IRF)1, and IRF8 (Ziegler et al., 2020). Other researchers predicted specificity protein 1 and HNF4 α as positive and negative regulators of *ACE2* gene transcription, and confirmed these predictions in immortalized human alveolar epithelial cells and the A549 lung adenocarcinoma cell line using small molecule inhibitors and siRNA in promoter reporter assays and protein immunoassays (Han et al., 2024). Interestingly, an earlier study had identified HNF4 α as an activator of *ACE2* gene transcription in the HepG2 human hepatoma cell line (Niehof and Borlak, 2011).

Several groups have investigated *ACE2* expression in human retinal cells (Yuan et al., 2020; Eriksen et al., 2021; Albertos-Arranz et al., 2023). Our observations made using multiple primary human retinal pigment epithelial cell isolates confirm the findings of studies undertaken with human adult eye cell isolates (Eriksen et al., 2021) and human retinal tissue sections (Albertos-Arranz et al., 2023), as well as a bioinformatics analysis of a range of public RNA-sequencing datasets (Yuan et al., 2020). Retinal pigment epithelial cells are readily isolated from non-diseased human cadaver eyes. Given the role of *ACE2* genotype in the susceptibility to SARS-CoV-2 infection (Hattori et al., 2022; Hu P. et al., 2022a), the availability of multiple individual isolates for research studies is important. We also noted differences in the level of *ACE2* gene expression between cell isolates. Although our work was not directed at retinal pathology in COVID-19, being primarily a respiratory disease, there are cases of retinopathy that suggest infection of the retinal pigment epithelium *in vivo* (Costa et al., 2021; Zhang and Stewart, 2021; Ng et al., 2024).

A novel short isoform of *ACE2* that lacks most of the SARS-CoV-2 binding site, and is inducible by interferon and by rhinovirus, has been described by three independent groups (Ng et al., 2020; Onabajo et al., 2020; Blume et al., 2021). Human primary bronchial

epithelial cells upregulate the transcript encoding the short *ACE2* isoform following treatment with type I and type II interferons, and lung carcinoma cell lines Calu-3 and A549 respond similarly to type I interferon (Blume et al., 2021; Scagnolari et al., 2021). While this transcript is detectable in nasopharyngeal swabs from SARS-CoV-2-infected patients, and it shows a moderate positive correlation with the level of interferon-stimulated gene 15 transcript in these samples, the function of the short isoform of *ACE2* remains unclear (Oliveto et al., 2022). Interestingly, the truncated transcript is present in hTERT-RPE1 cells, an immortalized human retinal pigment epithelial cell line, although these cells do not show stable protein expression (Blume et al., 2021). The primers used in the current study amplify a region common to known reference sequences for long and short *ACE2* transcript variants and therefore would not differentiate between those encoding the short and long isoforms.

In conclusion, transcriptional blockade is receiving renewed attention as a therapeutic modality, particularly in those settings where it can be applied directly to cells involved in a disease (Henley and Koehler, 2021; Su and Henley, 2021). In COVID-19, which affects the respiratory tract, a locally applied, epithelial cell-directed transcription blocking agent is feasible. We have demonstrated the potential to use human retinal pigment epithelial cells as a straight-forward testing system for evaluating transcriptional blockade targeting *ACE2*. These primary epithelial cell isolates are readily generated from human cadaveric donor eyes, which are widely collected for use in corneal transplantation surgery (Gain et al., 2016), and thus more available than non-diseased human lung tissue. Moreover, interindividual differences in gene expression are captured by using multiple primary cell isolates as opposed to a single cell line.

Data availability statement

The RNA-sequencing dataset used in this study is available in the NCBI Gene Expression Omnibus repository under accession number GSE132091: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132091>.

Ethics statement

The studies using primary human retinal cells were approved by the Southern Adelaide Clinical Human Research Ethics Committee (protocol 175.13). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation did not apply because the participants were deceased persons. Next-of-kin gave consent for the research use of eye tissue that would otherwise be discarded after removal of corneas for use in corneal transplantation surgeries.

Author contributions

LA: Conceptualization, Formal Analysis, Investigation, Methodology, Writing—original draft, Writing—review and editing. AL: Formal Analysis, Investigation, Methodology, Writing—original

draft, Writing–review and editing. YM: Investigation, Methodology, Writing–original draft, Writing–review and editing. AT: Formal Analysis, Investigation, Methodology, Writing–review and editing. BA: Conceptualization, Supervision, Writing–review and editing. SD: Conceptualization, Supervision, Writing–review and editing. MM: Conceptualization, Supervision, Writing–review and editing. JS: Conceptualization, Funding acquisition, Supervision, Writing–original draft, 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/fddsv.2024.1416728/full#supplementary-material>

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