MicroRNA-494 inhibits nerve growth factor-induced cell proliferation by targeting cyclin D1 in human corneal epithelial cells

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Abstract. Nerve growth factor (NGF) is expressed in the human corneal epithelium and stroma. It is an efficient therapy for human corneal ulcers caused by neurotropic disease. However, little is known about the molecular mechanism of NGF in healing human corneal epithelial diseases. Numerous microRNAs (miRNAs) are expressed in the cornea and miRNAs have important roles in regulating corneal development. In the present study, novel miRNA regulators were demonstrated to be involved in NGF-induced human corneal epithelial cell (hCEC) proliferation. NGF treatment significantly downregulated the expression of miRNA-494 in hCECs *in vitro*. Furthermore, miRNA-494 increased G1 arrest in the immortalized human corneal epithelial cell (ihCEC) line and suppressed cell proliferation. Accordingly, bioinformatics programs and luciferase reporter assay demonstrated that miRNA-494 directly targeted cyclin D1 by binding to a sequence in the 3'-untranslated region. In addition, overexpression of miRNA-494 decreased both basal and NGF-induced cyclin D1 expression. NGF treatment partially suppressed miRNA-494 expression and restored cyclin D1 expression. Furthermore, co-transfection of miRNA-494 with the cyclin D1 ORF clone partially restored cyclin D1 mRNA and protein expression. These findings indicate that miRNA-494 and its target cyclin D1 may be a crucial axis for NGF in regulating the proliferation of hCEC. Specific modulation of miRNA-494 in hCEC could represent an attractive approach for treating cornea epithelial diseases.

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Introduction

Nerve growth factor (NGF) belongs to the neurotrophin (NT) family. In addition to the two classes of well-known NGF transmembrane receptors, the high-affinity neurotrophin receptor neurotrophic receptor tyrosine kinase 1 (TrkA) and the low-affinity pan-NT receptor nerve growth factor receptor (p75NTR), discoidin domain receptor tyrosine kinase 1 (TrkE) was found to be a receptor of NGF (1,2). The binding of a neurotrophin to its receptor induces dimerization and phosphorylation that initiates a signal transduction cascade and ultimately leads to gene transcription. NGF is expressed in the human corneal epithelium (3) and stroma (4), together with its two corresponding receptors, TrkA and p75NTR. NGF activates the TrkA signaling pathway and promotes cell survival and proliferation, whereas the activation of p75NTR can result in apoptosis (5). Furthermore, NGF supports stem cell self-renewal in corneal epithelial progenitor cells (3,4), a phenomenon that has recently gained attention as an efficient therapy for human corneal ulcers caused by neurotropic disease (6).

MicroRNAs (miRNAs or miRs), a class of 19-25 nucleotide non-coding RNA molecules, negatively regulate the gene expression by binding to the 3'-untranslated region (3'-UTR) of mRNA, resulting in mRNA degradation or translational inhibition (7,8). Since miRNA can induce gene silencing with only partial sequence homology, miRNAs have more than one target. miRNA is important in multistep cellular functions and biological processes, including cell differentiation, proliferation, and apoptosis (9). In studies of diseases of the cornea, miRNA provides new insights into corneal epithelium physiology processes. Overexpression of miRNA-31 downregulates hypoxia-inducible factor 1 alpha inhibitor in human corneal epithelial cells (hCECs), thereby decreasing cellular glycogen and influencing their energy stores (10). miRNA-145 acts on human endothelin converting enzyme, decreases the expression of ITGB8 and promotes corneal epithelium differentiation (11). miRNA-205 directly targets SH2-containing inositol phosphatase-2 (SHIP2): The high-miRNA-205/low-SHIP2 phenotype of corneal epithelial cells is an innate mechanism to regulate

cell proliferation (12). In addition, numerous miRNAs are expressed in the cornea and have important roles in regulating corneal development (13,14). Peng *et al* (15) demonstrated that miRNA-103/107 can target the ribosomal kinase p90RSK2 and arrest cornea epithelial stem cells in G0/G1 stage, thereby contributing to a slow-cycling phenotype. Furthermore, miRNA-146a and miR-424 are involved in cornea epithelial cell migration and wound healing through targeting epidermal growth factor receptor and other signaling molecules such as interleukin-1 receptor-associated kinase 1 (16-19). These studies demonstrate the significance of miRNAs in corneal epithelial physiology.

In the present study, the miRNA signature in response to NGF treatment in hCECs was elucidated. These results indicated that NGF altered specific miRNA expression in hCECs, especially miRNA-494. miRNA-494, was downregulated in hCECs following NGF treatment, which decreased cyclin D1 expression, causing G1 arrest. Cyclin D1 was, furthermore, identified as a novel direct target of miRNA-494. Therefore, it appeared that miRNA-494 was an NGF-responsive miRNA in hCECs, which influenced NGF function in promoting corneal epithelial proliferation.

Materials and methods

Cell culture. hCECs were obtained from remaining eye tissues after the removal of the central corneal button for penetrating keratoplasty, at the eye bank of Shanghai Eye, Ear, Nose, and Throat Hospital (Shanghai, China). Cells were cultured as described in our previous study (20).

The immortalized human corneal epithelial cell (ihCEC) line was kindly provided by Dr Shi Wei-yun (Shandong Eye Hospital, Shandong Eye Institute, Shandong Academy of Medical Sciences, Jinan, China) and Professor Kaoru Araki-Sasaki (Osaka University, Osaka, Japan). The ihCEC line was maintained in DMEM/F12 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin. The cells were maintained in a humidified atmosphere at 37°C with 5% CO_2. The medium was changed every 3 days, and the cells were reseeded after reaching 80-90% confluence. The cells at passages 4-8 were used for experiments (21).

Immunofluorescence. ihCECs were seeded onto glass cover slips placed in a 24 well plate ($1x10^4$ cells/well). Coverslips were fixed in 4% paraformaldehyde for 10 min at room temperature. Prior to permeabilization at 4°C for 20 min in 0.5% Triton X-100 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), cells were washed 3 times in PBS. In order to avoid nonspecific binding, cells were treated with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at room temperature for 1 h, after which cells were incubated with primary antibodies against cytokeratin 12 (CK12; 1:200; cat. no. sc-17099; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C. After 4 washes with PBS for 5 min each, cells were incubated with fluorescein isothiocyanate-conjugated anti-goat secondary antibody (1:200; cat. no. F7367; Sigma-Aldrich; Merck KGaA) for 45 min. The cells were rinsed with PBS, then incubated with 4',6-diamidino-2-phenylindole (DAPI;

Sigma-Aldrich; Merck KGaA) at room temperature for 1 min. ihCECs were analyzed by fluorescence microscopy.

Drug treatment. Following 24 h incubation, human recombinant NGF-β (Sigma-Aldrich; Merck KGaA) diluted in PBS containing 0.1% bovine serum albumin, was added to the culture medium at a final concentration of 25 ng/ml. The cells were then continuously incubated for 1 h at 37°C. The control cells were incubated in fresh culture medium without the addition of NGF-β.

miRNA microarray analysis. The RNAs were obtained according to the TRIzol method. The cells were washed with cold 1X PBS 3 times, and the monolayer was covered with 1 ml of TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and lysed by repeated pipetting. Subsequently, the lysed samples were incubated on ice for 5 min and centrifuged for 15 min at 15,000 x g at 4°C. The supernatants were transferred into new tubes and 200 ml chloroform was added. The tubes were shaken vigorously by hand for 15 sec, and the mixture was incubated at 15-30°C for 2 min. Subsequently, the chloroform-mixed samples were centrifuged for 15 min at 15,000 x g at 4°C. The aqueous phase was transferred to other tubes and the RNA was precipitated from the aqueous phase by mixing with 500 ml isopropyl alcohol for incubation at 15-30°C for 10 min. The mixture was centrifuged for 30 min at 15,000 x g at 4°C and the RNA precipitate formed a gel-like pellet on the side of the tube at the bottom. The gel-like pellet was acquired via removal of the supernatant and washed once with 1 ml 75% ethanol, followed by centrifugation for 10 min at 10,000 x g at a temperature of 4°C. The pellet was dried and dissolved in RNase-free water. The RNAs were sent to the MicroRNA Microarray Service (Gminix Biologic Science Co., Ltd, Shanghai, China), which used Affymetrix GeneChip miRNA Array.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from hCECs using TRIzol, according to the manufacturer's instructions. RNAs were reverse transcribed to cDNA with an RT-PCR system. cDNA for miRNA qPCR was synthesized using a One Step PrimeScript® miRNA cDNA Synthesis kit (cat. no. D350A; Takara Biotechnology Co., Ltd., Dalian, China). The reaction conditions were as follows: 37°C for 60 min, followed by a final step of 37°C for 5 sec. cDNA for mRNA real-time PCR was synthesized using a PrimeScript™ RT reagent kit with gDNA Eraser (cat. no. RR047A; Takara Biotechnology Co., Ltd.). The following conditions were used: gDNA Eraser at 42°C for 2 min, cDNA synthesis at 37°C for 15 min, followed by 85°C for 5 sec. SYBR® Premix Ex Taq™ II Real-Time PCR kit (cat. no. DRR081A; Takara Biotechnology Co., Ltd.) was used with the ABI ViiA7 Real-Time PCR System (Thermo Fisher Scientific, Inc.) for qPCR analysis, which was performed as follows: Pre-denaturation at 95°C for 30 sec, 40 cycles of 95°C for 5 sec, followed by 60°C for 34 sec. The PCR products were stored at 4°C. The primers used are listed in Table I. The universal-miRNA qPCR primer (cat. no. D350) and miRNA-15, miRNA-184, miRNA-320c, U6RNA primers were purchased from Takara Biotechnology Co., Ltd. The ΔΔCq method (22) for relative quantitation of gene and miRNA expression was used to determine cyclin D1

Table I. Reverse transcription - quantitative polymerase chain reaction primer sequences.

Target gene	Primer sequence
U6	F-CAAATTCGTGAAGCGTT
	R-TCAACTGGTGTCGTGG
Cyclin D1	F-AGCCATGGAACACCAGCTC
	R-GCACCTCCAGCATCCAGGT
GAPDH	F-TGCACCACCAACTGCTTAGC
	R-GGCATGGACTGTGGTCATGAG
miRNA-494	F-TGAAACATACACGGGAAACCTC
	R-Universal-miRNA qPCR primer
miRNA-1305	F-TTTTCAACTCTAATGGGAGAGA
	R-Universal-miRNA qPCR primer

miRNA, microRNA; F, forward primer; R, reverse primer.

and miRNA-494 expression levels according to ABi protocol. U6 RNA and GAPDH were used to normalize the relative abundance of miRNA-494 and cyclin D1, respectively.

Prediction of the target gene of miRNA-494. Gene Ontology (GO; www. geneontology.org) analysis was used to select significant GO. GO may be used to analyze the function of interesting genes in biological processes, cellular components and molecular functions (23). mirBase (www.microrna .org/microrna/home.do), miRWalk (zmf.umm.uni-heidelberg .de/apps/zmf/mirwalk/index.html) and targetScan (www.targetscan.org/vert_71) bioinformatics software was used to predict the target gene of miRNA-494.

Western blot analysis. The cells were lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitors (Complete, Mini, EDTA-free; Roche Applied Science, Penzburg, Germany) and Phosphatase Inhibitor Cocktails 1 and 2 (Sigma-Aldrich; Merck KGaA) at 4˚C for 30 min. The cells were then centrifuged at 15,000 x g for 20 min at 4˚C to achieve a clear state. Cell lysates were separated by 10% SDS-PAGE and transferred onto Hybond-P polyvinylidene difluoride membranes (Merck KGaA). Membranes were blocked in 5% skimmed milk at room temperature, for 30 min, then incubated overnight at 4˚C with mouse anti-cyclin D1 (1:1,000; 37 kDa; cat. no. SC-8396; Santa Cruz Biotechnology, Inc.,) or rabbit anti-GAPDH (1:5,000; 37 kDa; cat. no. AB-P-R 001; Miao Tong Biological Science & Technology Co., Ltd., Shanghai, China) primary antibodies. Membranes were then incubated at room temperature for 1 h with anti-mouse (1:2,000; cat. no. 7076; Cell Signaling Technology, Inc., Danvers, MA, USA) or anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.), respectively. Immune reactive proteins were visualized using Enhanced Chemiluminescence Plus Western Blotting Detection reagents (GE Healthcare Life Sciences) and the LAS4000 luminescent image analyzer (Kodak, Rochester, NY, USA). Densitometric analysis of band intensity was performed using Carestream Molecular Imaging Software Standard Edition 5.0.2.30 (Kodak).

Virus and cyclin D1 ORF clone transfection. The lentiviral vector pGIPZ-GFP-miR-494 or negative control pGLV3-GFP-NC and lentiviral packaging plasmids (SunBio Medical Biotechnology, Shanghai, China) were co-transfected into 293FT packaging cells. The sequence of miR-494 was as follows: CAGTGCCATGTAGATTCGGGGAATCTT CCTGGAGGTGACACCAACATCGGCGAAGATTCGGCA GTTCTGTTTTGATTTTTTTTGTTTGTTTTTTGATCAGT GCTAATCTTCGATACTCGAAGGAGAGGTTGTCCGTG TTGTCTTCTCTTTATTTATGATGAAACATACACGGGA AACCTCTTTTTTAGTATCAAATCCCACCCTGGAGGC ACTTCCTGTTCCTGATGCAGCCTTCAGGGAGGGACG TTGCCGGCTCCACTCTCGGTAGGCGCAAGCGAGGCT GACAGCGTGCCATGC. The negative control sequence was as follows: TCTCGCTTGGGCGAGAGTAAGTAGTGAAGC CACAGATGTACTTACTCTCGCCCAAGCGAGA. The virus-containing supernatant was harvested and filtered 72 h post-transduction, then used to infect the ihCEC line with Polybrene® (Sigma-Aldrich; Merck KGaA) at a final concentration of 5 μg/ml. Following 24 h of transfection, the cell culture medium was replaced with fresh complete medium and the ihCEC line was incubated for an additional 48-72 h prior to determining the expression of green fluorescent protein (GFP). Following flow-cytometer (Epics Altra; Beckman Coulter, Inc., Brea, CA, USA) selection for GFP (+) cells, the stable clones were obtained. The analysis software used was Expo32 V1.2 Analysis Multicycle for Windows (Beckman Coulter, Inc.). Furthermore, pGIPZ-GFP-miR-494 ihCEC line was transfected with a cyclin D1 ORF clone in a pcDNA3.1 vector (GeneCopoeia, Inc., Rockville, MD, USA) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cells were cultured for 72 h following transfection.

Cell cycle analysis. A total of $5x10^6$ cells were harvested, washed twice with cold PBS, fixed overnight with 75% cold ethanol at 4˚C, then washed with PBS again. Cells were then resuspended in 0.2%-Triton X-100 containing 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase at 4˚C for 30 min in the dark. The pGIPZ-GFP-miR-494 or pGIPZ-GFP-NC ihCEC line was analyzed on FACS Calibur™ Flow Cytometer and Cell Quest™ software (BD Biosciences, San Diego, CA).

3'-UTR Construct. mirBase, miRWalk and targetScan bioinformatics software was used to predict the binding site between cyclin D1 and miRNA-494. The cyclin D1 3' UTR contains a putative binding site for miRNA-494 with the seed region at 1345-1364. The wild-type or mutant 3'-UTR fragment was cloned into the pmiRGLO reporter vector (Promega Corporation, Madison, WI, USA): The wild-type construct contained a 300 bp truncated portion of the wild-type 3' UTR, including the seed sequence; the mutant construct contained the same 300 bp truncated portion of the wild-type 3' UTR, but with the sequence at 1357-1363 -ATGTTTC-replaced by -AACTTAG-.

Luciferase reporter assay. 293T cells grown in 6-well plates ($1.0x10^5$ cells per well) were harvested for assays 48 h after transfection with pcDNA6.2-GW/miRNA-494 and pmiRGLO-cyclin D1-3' UTR vectors (Promega Corporation). Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher

Table II. Differential miRNA expression in HCEC following NGF treatment.

miRNA	Fold change (NGF/control)
Upregulated	
miR-3179	5
miR-598	2
miR-634	24
miR-129-3p	15
miR-211	31
miR-3115	2
miR-582-5	9
let-7b	29
miR-129	22
miR-1246	4
miR-17	2
miR-202	28
miR-297	2
miR-615-5p	4
miR-1181	7
miR-548b-3p	3
miR-146a	6
miR-199b-5p	24
miR-223	10
miR-495	3
miR-1183	25
miR-515-5p	13
miR-103	18
miR-1825	21
miR-18b	2
miR-217	4
miR-524-5p	16
Downregulated	
miR-1182	2
miR-1202	4
miR-1208	11
miR-1268	2
miR-1275	2
miR-1471	4
miR-15a	19
miR-188-5p	8
miR-204	3
miR-220b	2
miR-3127	5
miR-31-star	2
miR-337-5p	2
miR-889	2
miR-1207-5p	12
miR-1225-5p	3
miR-494	20
miR-1305	32
miR-150	24
miR-184	10
miR-19b	18
miR-219-2-3p	3
miR-30b	5
miR-3168	2
miR-320c	31
miR-342-5p	2

Table II. Continued.

miRNA	Fold change (NGF/control)
miR-513a-5p	33
miR-516a-5p	2
miR-555	2
miR-575	4
miR-630	5
miR-641	9
miR-647	3
miR-662	4
miR-765	5
miR-513b	24
miR-543	3
miR-568	2
miR-671-5p	11
miR-939	3
miR-660	2
miR-1274b	2
miR-483-3p	2
miR-646	16
miR-4297	2
miR-4260	2
miR-638	2
miR-371-5p	10
miR-370	22
miR-601	4

miRNA, microRNA; miR, microRNA; HCEC, human corneal epithelial cell; NGF, nerve growth factor.

Scientific, Inc.) was used for the corresponding NC. The firefly and *Renilla* luciferase activities were measured using a dual-luciferase reporter assay system (Promega Corporation) with a microplate luminometer, and each sample's luciferase activity was normalized to that of *Renilla*.

Statistical analysis. Each experiment was performed in triplicate. Data are presented as the mean ± standard deviation. Two group comparisons were analyzed using Student's t-tests; comparisons between ≥3 groups were performed using analysis of variance followed by Tukey's post hoc test. Analyses were performed using SPSS software, version 19.0 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Differential miRNA expression in hCECs following NGF treatment. To identify the expression of miRNA in hCECs exposed to NGF, an Affymetrix GeneChip miRNA Array was performed in hCECs following NGF treatment. Differential expression was defined using a cut-off value of 2-fold change. A total of 27 miRNAs were upregulated and 50 miRNAs were downregulated in NGF-treated hCECs (Table II). Since the expression of cyclin D1 is increased following NGF treatment in hCECs (20), whereas miRNA had a negative regulatory
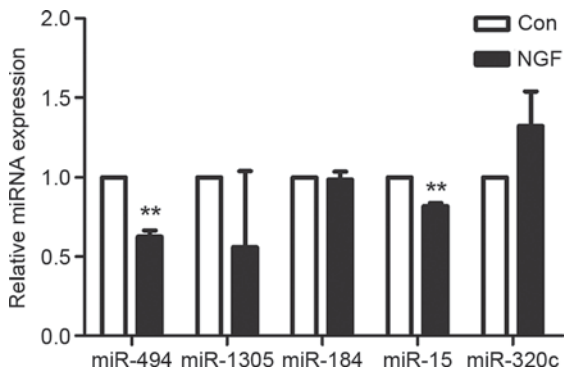
Figure 1. miRNA-494 expression is repressed by NGF treatment in hCEC. Reverse transcription-quantitative polymerase chain reaction analysis demonstrated the expression levels of miRNA-494, miRNA-1305, miRNA-184, miRNA-15 and miRNA-320c in human corneal epithelial cells following 1 h treatment with 0 or 25 ng/ml NGF relative to expression of U6 control. **P<0.01 vs. untreated control. miRNA, microRNA; NGF, nerve growth factor; Con, untreated control.

effect on its target genes (24), the present study focused on the downregulated miRNAs. GO analysis was performed on the downregulated miRNAs, and 5 miRNAs (miRNA-494, miRNA-1305, miRNA-15, miRNA-18, and miRNA-320c), which functioned on cell proliferation, were selected for further investigation. The microarray results were then validated by RT-qPCR. Consistent with the microarray data, miRNA-494, miRNA-1305 and miRNA-15 were demonstrated to be downregulated in NGF-treated hCECs to 62.1% (P=0.005), 56.0% (P=0.062), and 81.7% (P=0.003) of the levels of untreated controls, respectively (Fig. 1). The effect on miRNA-494 was the most significant, therefore, miRNA-494 was chosen as a candidate for further study.

Overexpression of miRNA-494 by transfecting lentivirus pGIPZ-GFP-miR-494 in the ihCEC line. ihCECs were demonstrated to express CK12, which is a biomarker for the corneal epithelium (Fig. 2). The ihCEC line was transfected with lentivirus pGIPZ-GFP-miR-494. The negative control (NC) received pGLV3-GFP-NC (Fig. 3A and B). Flow cytometry was utilized after 72 h to enable sorting of the GFP-positive cells to establish the pGIPZ-GFP-miR-494 ihCEC and pGLV3-GFP-NC ihCEC lines, which contained GFP (+) cells up to 98% (Fig. 3C). The expression of miRNA-494 was 3.60 times greater in the pGIPZ-GFP-miR-494 ihCEC line than in the NC line (P<0.001; Fig. 3D).

miRNA-494 increases G0/G1 arrest in the ihCEC line. To investigate the role of miRNA-494 in the ihCEC line, the cells were treated with NGF and the cell cycle distribution analyzed by flow cytometry (Fig. 4A). Overexpression of miRNA-494 increased the cell number in the G1 phase, compared to the pGLV3-GFP-NC ihCEC lines (Fig. 4B). NGF treatment, which partially reversed the negative effect of the miRNA-494, promoted cell cycle progression by decreasing cell distribution in the G1 phase (Fig. 4B). Overall, these results suggested that miRNA-494 inhibited NGF-induced proliferation by inhibiting cell cycle progression from the G0/G1 phase into the S phase in the ihCEC line.

miRNA-494 directly targeted the cyclin D1 gene. To clarify the mechanism by which miRNA-494 suppressed cell proliferation, we first applied three bioinformatics prediction programs (miRanda, miRWalk, and TargetScan) to predict the potential target of miRNA-494. A binding site between miRNA-494 and cyclin D1 (gene ID: 595) was identified. As presented in Fig. 5A, the 3'-UTR domain of the cyclin D1 mRNA was demonstrated to be partially complementary to miRNA-494. A luciferase reporter system in which the wild-type (WT) or mutant (MUT) cyclin D1 3'-UTR was cloned into a luciferase expression vector was then utilized. The data demonstrated that miRNA-494 significantly inhibited the luciferase activity of cyclin D1 3'-UTR wild type in the 293T cells (P<0.001; Fig. 5B, white bars), whereas it had no significant effect on the mutant cells (P>0.05; Fig. 5B, black bars). Collectively, these results demonstrated that miRNA-494 directly targeted cyclin D1 mRNA by binding to its 3'-UTR.

miRNA-494 expression is decreased and cyclin D1 expression is increased by treatment with NGF. Cyclin D1 is important in cell cycle progression (25). To further substantiate the role of miRNA-494 in hCEC cell proliferation following exposure to NGF, miRNA-494 and cyclin D1 expression was examined in response to NGF. As determined by RT-qPCR analysis, in untreated cells, miRNA-494 was significantly overexpressed in the pGIPZ-GFP-miR-494 ihCEC line compared with the pGIPZ-GFP-NC ihCEC line (P<0.001; Fig. 6A). However, this effect was significantly attenuated by treatment with NGF (Fig. 6A, black bars). Cyclin D1 expression was upregulated by treatment with NGF (Fig. 6B, white vs. white bars and black vs. black bars). Overexpression of miRNA-494 decreased NGF-induced cyclin D1 mRNA (Fig. 6B) and protein expression (Fig. 6C), as demonstrated by RT-qPCR and western blot analysis, respectively. Furthermore, transfection of cyclin D1 ORF clone in the pGIPZ-GFP-miR-494 ihCEC line resulted in upregulation of cyclin D1 mRNA (Fig. 7A, 2nd vs. 3rd bars) and protein expression (Fig. 7B, 2nd vs. 3rd lanes). These changes demonstrated the specific functions of miRNA-494, in mediating the expression of the cell cycle-associated gene cyclin D1 and hCEC proliferation.

Discussion

Although many studies have focused on the role of NGF in healing human corneal epithelial diseases (26), the molecular mechanism remained elusive. In the present study, miRNA-494 was demonstrated to be significantly downregulated in NGF-treated hCECs and was demonstrated to target cyclin D1 by binding to its 3'-UTR. Overexpression of miRNA-494 confirmed its anti-proliferation function by attenuating cyclin D1 expression and inducing G1 arrest *in vitro*. In order to further confirm the possible link between miRNA-494 and cyclin D1, cyclin D1 ORF clone was transfected into the pGIPZ-GFP-miR-494 ihCEC line and cyclin D1 expression was investigated. NGF treatment decreased the expression of miRNA-494 and restored cyclin D1 expression, leading to hCEC proliferation. In our previous study, we demonstrated that NGF regulated hCEC proliferation via the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase
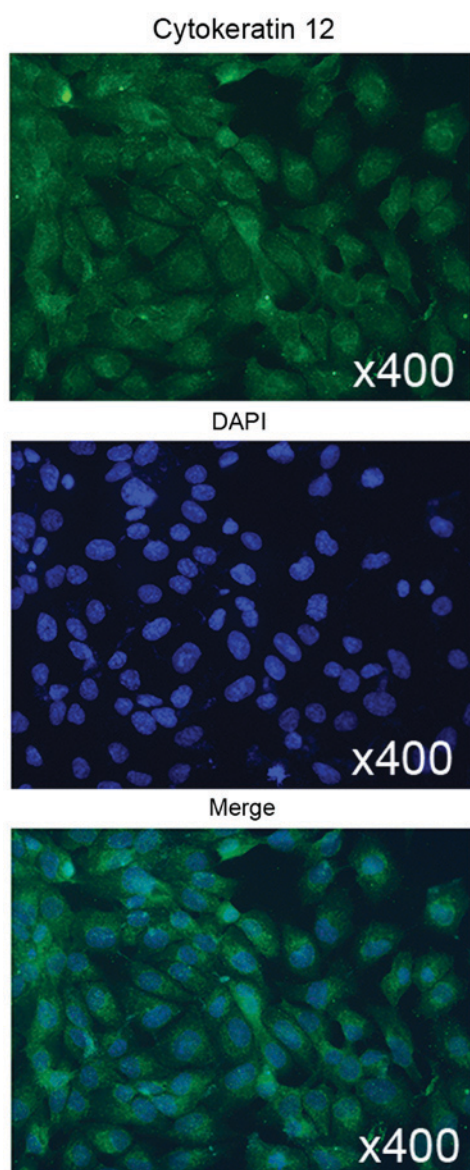
Figure 2. Immunofluorescence analysis of cytokeratin 12 (green) and DAPI (blue) in immortalized human corneal epithelial cells.
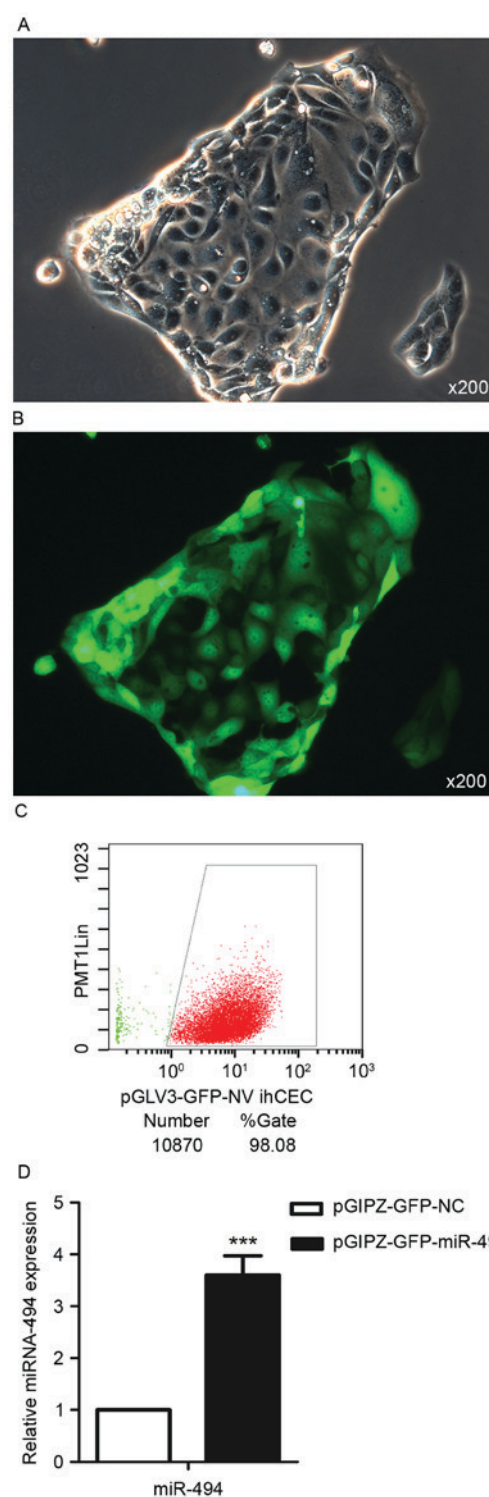

Figure 3. Overexpression of miRNA-494 in lentivirus pGIPZ-GFP-miR-494 ihCECs. The lentivirus pGIPZ-GFP-miR-494 ihCEC line was examined using (A) phase contrast microscopy and (B) fluorescent microscopy. (C) Flow cytometry was used to sort the GFP (+) cells to establish the pGIPZ-GFP-miR-494 ihCEC line and the pGLV3-GFP-NC ihCEC line, which contained the GFP (+) cells at concentrations up to 98%. (D) miRNA-494 expression in the pGIPZ-GFP-miR-494 and pGIPZ-GFP-NC ihCEC lines was measured using reverse transcription-quantitative polymerase chain reaction, with quantification relative to the U6 control. ***$P<0.001$ vs. pGLV3-GFP-NC. miRNA, microRNA; ihCEC, immortalized human corneal epithelial cell; GFP, green fluorescent protein; NC, negative control.

(ERK) signaling pathways. Furthermore, this research provides cognate evidence that miRNA-494 and its target gene, cyclin D1, could be a new crucial axis for NGF in regulating the proliferation of hCEC and treating corneal epithelial cell diseases (Fig. 8).

The human cornea produces and stores NGF at a concentration of 1,154±376 pg/mg under normal physiological conditions (21). Following the introduction of a lesion to corneal tissue, the concentration of NGF increased to a maximum concentration of 3,010±284 pg/mg 48 h after injury (27). NGF not only stimulates corneal epithelial proliferation *in vivo* and *in vitro* (28), but also helps to maintain limbal epithelial stem cells (27,29). Endogenous NGF accelerates the corneal epithelial healing progress following cataract surgery (26,29,30), and topical administration of NGF enhances the neurotropic corneal ulceration healing process (31-33) in normal and diabetic patients (28). NGF promotes corneal wound healing by upregulating corneal sensitivity and improving tear function (29). Inhibiting the pathway of endogenous NGF by

Ab-NGF or NGF mutational receptor suspends the epithelial healing process and results in corneal opacification (34,35).
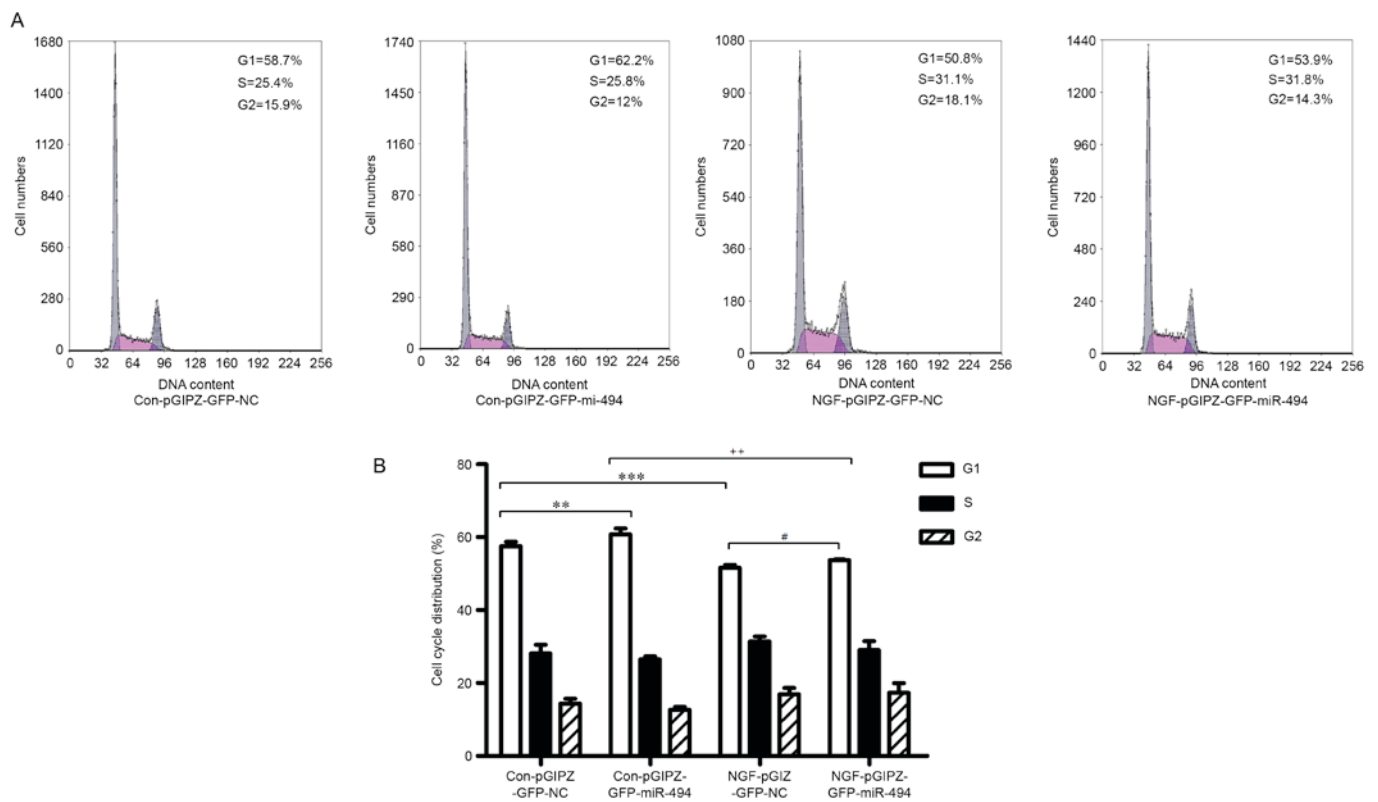
Figure 4. miRNA-494 increases G1 arrest. The ihCEC line was transfected with pGIPZ-GFP-miR-494 or pGIPZ-GFP-NC, treated with 0 (con) or 25 ng/ml NGF for 1 h. (A) Cell cycle analysis was then performed and (B) the percentage of cells in each cell cycle phase was calculated. *P<0.05, **P<0.01, ***P<0.001, #P<0.05 and ++P<0.01, as indicated by brackets.

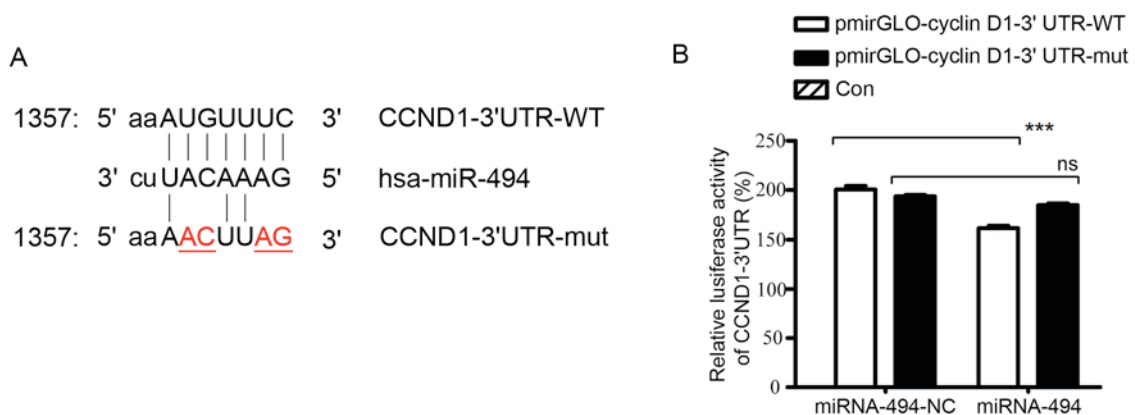Figure 5. miRNA-494 directly targets the cyclin D1 gene. (A) Putative binding site of miRNA-494 and WT cyclin D1 3'-UTR, and mutated cyclin D1 3'-UTR binding site in which mutated bases are shown in red and underlined. (B) Luciferase reporter assay of 293T cells transfected with pmiRGLO-cyclin D1-3' UTR-WT or pmiRGLO-cyclin D1-3' UTR-mut together with miRNA-494 or NC. ***P<0.001. miR, microRNA; hsa, *Homo sapiens*; WT, wild type; UTR, untranslated region; mut, mutated; NC, negative control; ns, not significant.

Although many studies have investigated the function of NGF on hCEC proliferation, the mechanism has remained inconclusive.

miRNAs have previously been recognized as regulators of cell proliferation during NGF treatment. Zhang *et al* (36) reported that expression of *Rattus norvegicus*-miR-541 was downregulated by NGF stimulation in PC12 cells and that it targeted Synapsin-I, leading to neuritogenesis. Furthermore, miRNA-221 and miRNA-222 were upregulated by NGF in

PC12 cells (33). Thus far, the role of miRNA in NGF-induced hCEC proliferation had not been described. To the best of our knowledge, the present study demonstrates for the first time that miRNA-494 is an NGF-responsive miRNA in hCEC proliferation.

miRNA-494, which is located on chromosome 14q32.31, has profound impacts on many biological processes. It has been reported to regulate both anti-proliferation and pro-proliferation proteins. For instance, in human hepatocellular carcinoma,
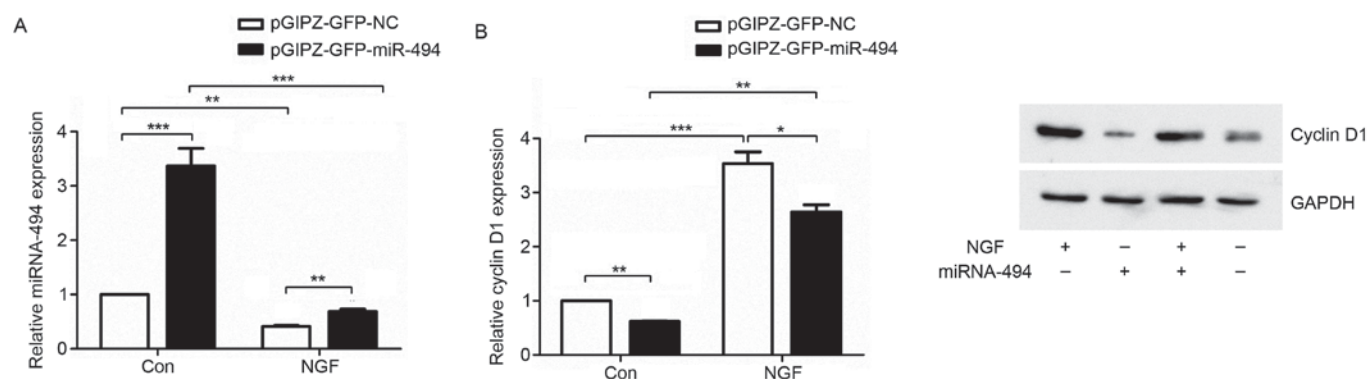
Figure 6. miRNA-494 is NGF-responsive and overexpression of miRNA-494 decreases cyclin D1 expression. (A) RT-qPCR was performed to determine the expression of miRNA-494. Transfecting pGIPZ-GFP-miR-494 caused the miRNA-494 levels to increase compared with transfection with pGIPZ-GFP-NC, whereas NGF treatment attenuated the miRNA-494 expression. (B) Overexpression of miRNA-494 decreased both cyclin D1 mRNA and protein expression in ihCECs, as demonstrated by RT-qPCR and western blot analysis, respectively. NGF treatment inhibited miRNA-494 expression and increased cyclin D1 expression. U6 was used as an internal control for RT-qPCR and GAPDH was used as an internal control for western blots. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, as indicated by brackets. miRNA, microRNA; NGF, nerve growth factor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control; ihCECs, immortalized human corneal epithelial cells.
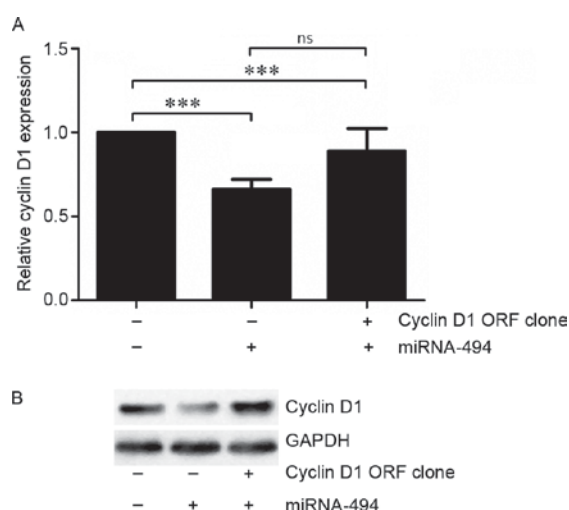

Figure 7. Cyclin D1 ORF clone upregulates cyclin D1 expression in pGIPZ-GFP-miR-494 ihCECs. ihCECs were transfected with pGIPZ-GFP-miR-494 or cyclin D1 ORF clone. Post-transfection (72 h), cyclin D1 (A) mRNA and (B) protein expression levels were determined by RT-qPCR and western blot analysis, respectively. Cyclin D1 ORF clone rescued cyclin D1 expression in pGIPZ-GFP-miR-494 ihCECs. U6 was used as an internal control for RT-qPCR and GAPDH was used as an internal control for western blots. $^{***}P<0.001$. ORF, open reading frame; miRNA, microRNA; ihCECs, immortalized human corneal epithelial cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ns, not significant.
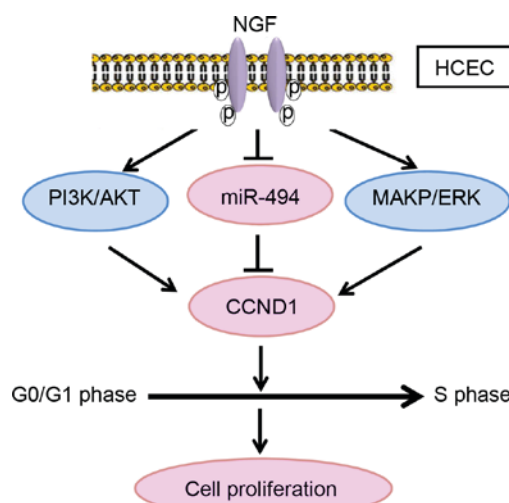

Figure 8. Model of NGF regulation of hCEC proliferation via PI3K/Akt and MAPK/ERK signaling pathways and miR-494 and cyclin D1. NGF, nerve growth factor; hCEC, human corneal epithelial cell; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; miR, microRNA; CCND1, cyclin D1.

increasing miRNA-494 expression promotes cell proliferation by advancing the G1/S cell cycle transition and targeting the mutated genes in colorectal cancer tumor suppressor (37). However, Kim *et al* (38) demonstrated that miRNA-494 represses gastrointestinal stromal tumor by negatively regulating KIT, an oncogenic tyrosine kinase. Furthermore, miRNA-494 was shown to target insulin like growth factor 2 mRNA binding protein 1 to suppress cell proliferation in A549 cells (39). The present study investigated the function of miRNA-494 in hCECs and demonstrated that miRNA-494 was downregulated in NGF-treated hCECs. Furthermore, ectopic miRNA-494 suppressed cell proliferation by inhibiting cyclin D1 expression, but this anti-proliferative activity was partially reversed by NGF treatment. This could be explained by the following: one miRNA could regulate approximately 200 kinds of mRNA due to the allowance of a mismatched base pair with mRNA (40). Therefore, miRNA-494 might function in either a pro-proliferation or anti-proliferation manner according to the cellular environment and cell-specific targets. Although the present study indicated that the downregulation of miRNA-494 was associated with NGF treatment in hCECs, little is known about the mechanism of the regulation of miRNA-494 expression; this should be examined in future studies.

Several miRNA-494 targets have been recently identified in various cellular systems, including activating transcription factor 3 (41), phosphatase and tensin homolog (42), Bcl-2-like protein 11 (43), nucleolin (44) and solute carrier family 26 member 3 (45). In addition, miRNA-494 was

reported to target mitochondrial transcription factor A and Forkhead box j3 in murine myoblast C2C12 cells (46). The present study identified cyclin D1 as a novel target of miRNA-494 in hCEC proliferation. Cyclin D1, which is a member of the D-type cyclins (D1, D2, and D3) family, interacts with cyclin-dependent kinase 4 (CDK4) (47,48) to form a cyclin D1/CDK4 complex. The activation of CDK4 promotes cell cycle progression from G1 to S phase (49,50) through phosphorylating substrates and transcription factors, which have roles in proliferation and differentiation. Cyclin D1 is regulated by numerous miRNAs, including *Homo sapiens (hsa)*-miR-638 (51), hsa-miR-425, hsa-miR-152, hsa-miR-24 (52), and hsa-miR-21 (53). The data in the current study demonstrated that cyclin D1 was the target of miRNA-494 and was downregulated in cells overexpressing miRNA-494. Consequently, the cell cycle was arrested in the G1 phase and cell proliferation was inhibited.

In summary, the present study demonstrated that NGF significantly downregulated the expression of miRNA-494 in hCECs *in vitro* and miRNA-494 overexpression suppressed cell proliferation by directly binding to the 3' UTR of cyclin D1. Therefore, miRNA-494 may represent a promising therapeutic target for treating corneal epithelial diseases.

Acknowledgements

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