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Effects of lentiviral short hairpin RNA silencing of Toll-like receptor 4 on the lens epithelial cell line HLEC

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ABSTRACT. The aim of this study was to observe the proliferation of, and cell-cycle changes in, the human lens epithelial cell line HLEC after Toll-like receptor 4 (*TLR4*) gene silencing. HLEC cells were transfected with four TLR4-short hairpin RNA (shRNA) lentiviral vectors or the control lentivirus (pGCL-GFP-shRP-1, -2, -3, -4, NC). *TLR4* silencing was verified in these cells 96 h post-transfection using real-time polymerase chain reaction and western blot. We also observed the change in number of pGCL-GFP-shRP-4-transfected HLEC cells with silenced *TLR4* (multiplicity of infection = 10). Cell proliferation was analyzed 48 h after transfection by a standard Cell Counting Kit-8 (CCK-8) assay, and the cell cycle changes were detected by flow cytometry. The number of cells with silenced *TLR4* decreased with time. The decrease in TLR4 expression led to decelerated cell proliferation. Cells with silenced TLR4 (for 48 h) were arrested in the G1 phase; that is, the cell cycle was prolonged and cell division was decelerated. Lentivirus-mediated RNA interference effectively silenced

TLR4 expression in HLEC cells, which decelerated their proliferation rate and extended the cell cycle.

Key words: Lentivirus; Toll-like receptor 4; Cell apoptosis

INTRODUCTION

Posterior capsule opacification has been attributed to rapid proliferation and epithelial-mesenchymal transition of residual lens epithelial cells after surgery; however, the mechanism underlying posterior capsule opacification remains unclear. Human lens epithelial cells (HLEC) have typical epithelioid morphology and are plump and closely connected under normal conditions; these cells, when stimulated by surgery, change substantially because of the continuous release of inflammatory factors such as transforming growth factor beta. The cells that are originally shaped oval polygon gradually elongate, flatten, and aggregate because of the loss of intercellular connections. Meanwhile, the G1 phase of the cell cycle is shortened, accompanied by accelerated division and proliferation. Toll-like receptor (TLR), the earliest discovered lipopolysaccharide receptor, is expressed in immune cells and many tumor cells (Rakoff-Nahoum and Medzhitov, 2008). TLR4 is a portal protein that initiates an inflammatory chain reaction in the human body, which promotes proliferation and leads to resistance to apoptosis in tumor cells (Rakoff-Nahoum and Medzhitov, 2009). In this study, the normal HLEC were transfected with short hairpin RNA (shRNA)-TLR4 using a lentiviral vector to observe their proliferation after TLR4 silencing.

MATERIAL AND METHODS

Materials

The HLEC was purchased from China Center for Type Culture Collection. Four groups of TLR4-shRNA lentiviral vectors and control lentivirus (pGCL-GFP-shRP-1, -2, -3, -4, NC) were provided by the Department of Ophthalmology at our hospital. The fluorescence microscope was purchased from Olympus (Tokyo, Japan). The ABI-7500 real-time PCR system was obtained from Applied Biosystems (Foster City, CA, USA). The automatic microplate reader and flow cytometer were purchased from BD Biosciences (Franklin Lakes, NJ, USA). The Cell Counting Kit-8 (CCK-8) kit was obtained from Dojindo Molecular Technologies, Ltd. (Rockville, MD, USA). A standard western blot kit was purchased from Amersham Biosciences (Little Chalfont, UK). The SYBR master mix was purchased from TaKaRa (Otsu, Japan). Dulbecco's modified Eagle's medium, trypsin, glutamine, and fetal bovine serum were obtained from Gibco (Waltham, MA, USA). Dimethyl sulfoxide was obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-TLR4 (1:1000 dilution), anti-β-actin (1:1200 dilution), and secondary antibodies were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA). The primers were synthesized by Tiangen Biotechnology (Tiangen, China).

Cell transfection

shRNA provides specific, long-lasting gene silencing. The shRNA is transcribed to the cell nucleus after the vector is integrated into the host genome through polymerase II or

polymerase III. In this study, the cells were cultured in normal culture medium for 24 h until 35-45% of them fused together upon the addition of an appropriate amount of lentivirus. The cells were again cultured in normal culture medium after 12 h. Green fluorescent protein (GFP)-tagged gene expression was observed under a fluorescence microscope 4-6 days after transfection, and cells with a transfection efficiency >70% were selected for subsequent analyses.

Detection of interference efficiency by real-time PCR

The cells were collected and centrifuged at 1000 rpm, and the supernatant was discarded. Then, 1 mL TRIzol solution was added to the precipitate and the mixture was gently blown, left still for 5 min at room temperature, and transferred to 1.5-mL Eppendorf tubes. The residue was mixed with 200 μL chloroform, left still for 15 min at room temperature, and subsequently centrifuged. The supernatant was collected and an equal volume of pre-cooled isopropanol at -20°C was added; the mixture was precipitated at -20°C for 10 min and centrifuged. The supernatant was thereafter discarded. The precipitate was washed with 750 mL/L ethanol, centrifuged, dried at room temperature, and completely dissolved by adding RNase-free water. The concentration of extracted RNA was determined by UV-vis spectroscopy. The extracted RNA was reverse-transcribed to cDNA, treated with SYBR Master Mix and subjected to real-time PCR on ABI-7500 real-time PCR system.

Detection of TLR4 protein expression by western blot

The culture medium was removed and pre-cooled lysis buffer was added to the cells. The cells were scraped off with a pre-cooled scraping knife, lysed on ice for 10-15 min, and centrifuged. The supernatant was thereafter collected, mixed with 6X loading buffer, and boiled at 100°C for 5-10 min. The protein samples were then separated by SDS-PAGE and electronically transferred to a polyvinylidene difluoride membrane. The membrane was then blocked in Tris-buffered saline containing Tween-20 at room temperature for 1 h, incubated with diluted primary antibodies against TLR4 or the internal reference for 2 h, washed, incubated with the corresponding horseradish peroxidase-labeled donkey anti-mouse secondary antibodies at room temperature for 2 h, washed again, and color-developed using the standard western blot kit.

Detection of cell proliferation by CCK-8 assay

CCK-8 assay is a sensitive colorimetric method for the determination of cell viability in proliferation assays. The cells were collected on 96-well plates (concentration >5000 cells/mL) and cultured for 2-3 days. The cells were again cultured with an equal volume of the culture medium, and 10 μL CCK-8 solution was added; the cells were incubated at 37°C for 1-2 h. The absorbance was measured at 450 nm using an automatic microplate reader to observe the speed of proliferation.

Detection of cell cycle by flow cytometry

Cells (1-5 x 10^6 cells/mL) were collected, centrifuged, and the culture medium was

discarded. The cells were washed with PBS, fixed in 700 mL/L ethanol at low temperature for 2 h and centrifuged. The fixing solution was discarded. The cells were then resuspended in PBS, stained with 1 mL propidium iodide staining solution for 30 min, and detected by flow cytometry.

RESULTS

Effects of TLR4 shRNA lentiviral vector on endogenous TLR4 expression in HLEC cells

A multiplicity of infection (number of lentivirus/number of cells) value of 10 was used based on the results of pre-experiment studies. HLEC cells were divided into a normal control group (NC) and four experimental groups (pGCL-GFP-shRP-1, -2, -3, and -4), and transfected on six-well plates.

The TLR4 mRNA and protein expression in each well was detected by real-time PCR and western blot, respectively, 96 h after transfection. Both experiments revealed that the target gene *TLR4* was silenced, particularly in pGCL-GFP-shRP-1, -2, and -4 (Figure 1). pGCL-GFP-shRP-4 was chosen for future experiments because of its high interference efficiency.

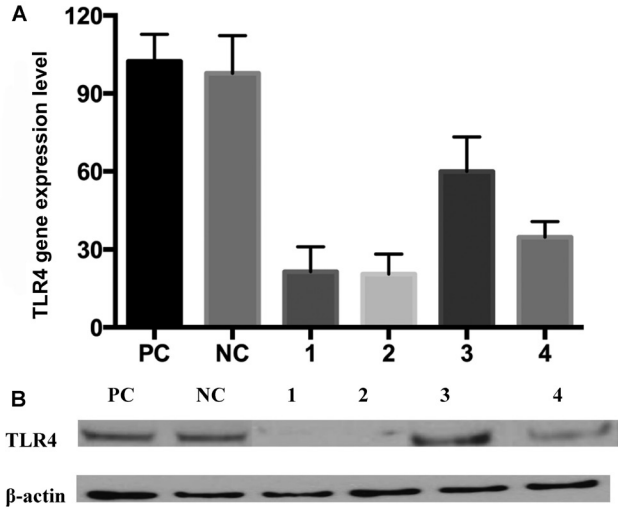

Figure 1. Interference efficiency 96 h post-transfection. **A.** *TLR4* gene expression in HLEC cells detected by real-time PCR. **B.** TLR4 protein expression detected by western blot. PC = blank cell group; NC = negative control group; *lane 1* = pGCL-GFP-shRP-1 group; *lane 2* = pGCL-GFP-shRP-2 group; *lane 3* = pGCL-GFP-shRP-3 group; *lane 4* = pGCL-GFP-shRP-4 group.

Observation of cell growth and transfection efficiency by fluorescence microscopy

The growth and transfection efficiency of cells transfected with pGCL-GFP-shRP-4 for 24 h were observed under a fluorescence microscope. All cells expressing the GFP showed good growth (Figure 2), with transfection efficiency >90%.

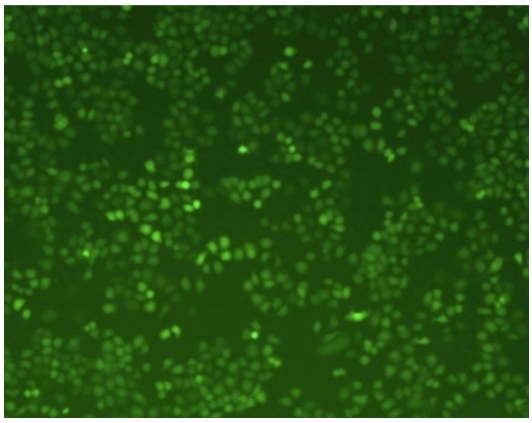

Figure 2. Fluorescence microscopy results of HLEC cells 24 h after transfection (100X).

Effects of TLR4 shRNA lentiviral vector on proliferation and cell cycle of HLEC cells

Cell culture results

pGCL-GFP-shRP-4 was then used in RNA interference (RNAi) of the cells. The number of cells transfected with lentiviruses decreased with time, while the number of cells transfected with NC did not change significantly (Figure 3).

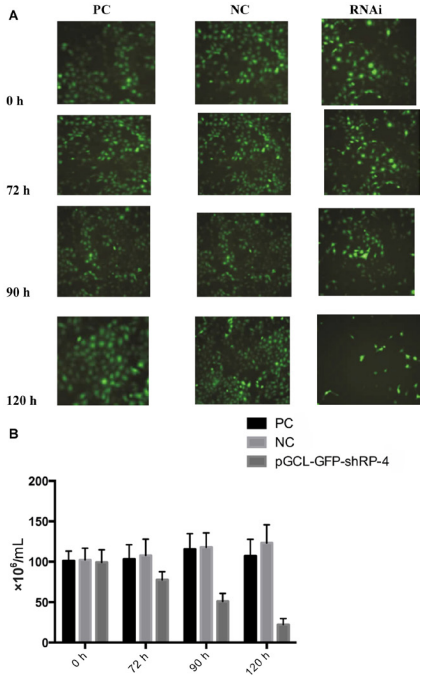

Figure 3. HLEC cell count after lentiviral vector treatment. **A.** Results of fluorescence microscopy at various time points after treatment. **B.** Cell counts in five randomly selected visual fields. PC = blank cell group; NC = negative control group.

CCK-8 assay results

The proliferation of TLR4-silenced cells was analyzed by detecting the optical density at 450 nm (OD_{450}) 1 and 2 h after the CCK-8 test. The experiment was performed in triplicate. One-way analysis of variance indicated that the cell proliferation was significantly inhibited ($P < 0.05$). The Dunnett test revealed that the control and experimental groups had significantly different outcomes ($P < 0.05$), suggesting that the decrease in TLR4 expression decelerated cell proliferation (Figure 4).

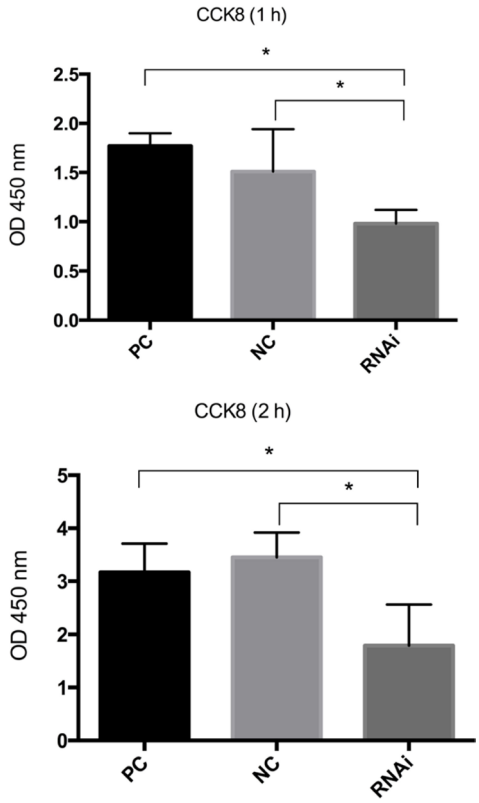

Figure 4. Detection of HLEC cell proliferation after *TLR4* silencing by CCK-8 assay (*vs* PC/NC, *$P < 0.05$). PC = blank group; NC = negative control group; RNAi = RNA interference group.

Flow cytometry results

The cells subjected to RNAi were significantly arrested in the G1 phase 48 h after transfection ($P < 0.05$). The experiment was performed in triplicate. The Dunnett test showed that the control and RNAi groups had significantly different outcomes ($P < 0.05$), indicating that absence of TLR4 arrested the cells in the G1 phase and prolonged the cell cycle. This caused the cell division to decelerate (Figures 5 and 6).

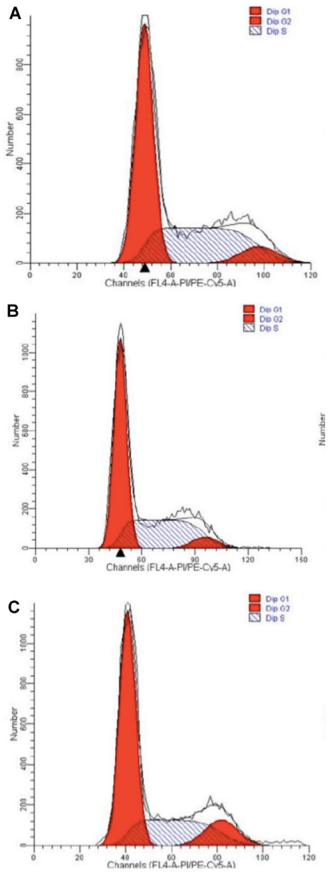

Figure 5. Flow cytometry results of HLEC cells 48 h after TLR4 silencing. **A.** Blank group. **B.** NC group. **C.** RNAi group.

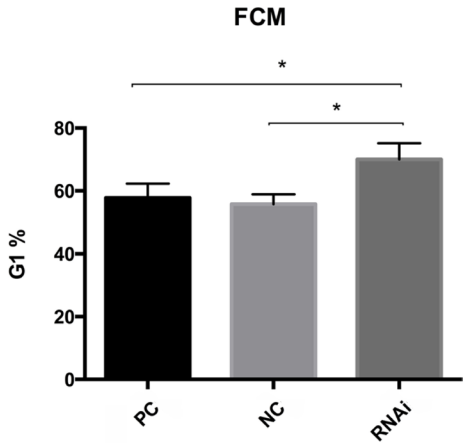

Figure 6. Cell cycle changes in *TLR4* RNAi HLEC cells (*vs* PC/NC, *P < 0.05). PC = blank cell group; NC = negative control group; RNAi = RNA interference.

DISCUSSION

TLR proteins play important roles in cell growth and proliferation. Disrupted TLR expression could lead to cancer, diabetes mellitus, obesity, cardiovascular disease, age-related disease, or other abnormally proliferative diseases. Activation of TLRs and gliocytes induces a central inflammatory response and neurodegeneration (Okun et al., 2009; Owens, 2009; Arroyo et al., 2011). TLRs are mainly expressed in the microglias and astrocytes in the nervous system (Kielian, 2006; Carpentier et al., 2008; Lehnardt, 2010). TLRs are also expressed in the neurons, photoreceptors, and pigment epithelial cells (Kindzelskii et al., 2004; Kigerl et al., 2007; Tu et al., 2011); therefore, they play a role in functions other than the control of host defense and response. TLRs play crucial roles in cell apoptosis, migration, and differentiation, as well as post-trauma repair. Chakravarty and Herkenham (2005) reported that TLR4 is involved in inflammatory response and autoimmune diseases of the central nervous system. In this study, TLR4 expression was inhibited in HLEC cells using a TLR4 shRNA lentiviral vector, in order to analyze the changes in cell proliferation after TLR4 silencing.

The effective target for *TLR4* RNAi was first screened from among four candidates. HLEC cells were collected from 5- to 12-month-old children who underwent surgeries for retinopathy, and transfected at the 3rd passage (when 60% of the cells were fused). This cell line can be continuously passaged until loss of proliferative capacity. Both real-time PCR and western blot analyses showed that the four shRNA interference lentiviruses, especially pGCL-GFP-shRP-1, -2, and -4, were capable of disrupting TLR4 expression in HLEC cells. The most effective lentivirus, pGCL-GFP-shRP-4, was selected for RNAi. The green fluorescence observed 24 h after transfection indicated the successful transfection of HLEC cells by this lentivirus.

The proliferation of HLEC cells was inhibited after transfection with a lentiviral vector. The results of flow cytometry indicated that although the proliferative capacities of RNAi and NC groups were not reduced, cell apoptosis was triggered as a result of TLR4 silencing. The CCK-8 assay revealed a reduced HLEC cell proliferation speed. The activation of some proteins in human lens epithelial cells may in turn accelerate the synthesis of related proteins, thereby speeding up cell division and proliferation (Lin et al., 2009; Yang et al., 2013). A reduction in the cell division and proliferation speeds can effectively delay the progression of posterior capsule opacification and maintain the transparency of the refractive medium (Li et al., 2011). Several other cell types with inhibited TLR4 were also arrested in the G1 phase in previous studies; as a result, these cells did not synthesize new DNA strands or produce the related proteins (Viallard et al., 2001; Bertoli et al., 2013), and the cell cycle remained incomplete. The inhibition of TLR4 expression prolongs the cell cycle, decelerates cell proliferation, and maintains the relative stability of epithelial cells. However, its mechanism of action remains to be elucidated.

shRNA are highly specific RNA that can accurately treat diseases without apparent side effects. Lentiviral packaging has become a mature and ideal genetic engineering technology after years of optimization and improvement (Moore et al., 2010). In this study, shRNA was successfully used to inhibit the gene expression *in vitro*; therefore, the specific inhibition of *TLR4* in human lens epithelial cells via shRNA transfection led to a reduction in cell proliferation and mesenchymal transition speed. However, further in-depth studies using animal models must be conducted to test the efficacy of this method *in vivo* with tolerable side effects, as well as to identify possible lentivirus-induced gene mutations in the host.

Conflicts of interest

The authors declare no conflict of interest.

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