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Effects of transfection on inflammatory factor production in LPS-induced HBE cells

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Summary. Transfection is an experimental technique typically used in biological experiments. In this study, we verified whether this technique may cause the release of inflammatory cytokines and affect cell viability. We used lipopolysaccharide (LPS)-induced human bronchial epithelial (16-HBE) cells as a model to evaluate whether cell transfection with Lipofectamine 3000 would affect LPS-induced inflammatory factors in HBE cells. MicroRNA (miRNA) negative control (NC)- and miR-584-mimics were transfected into 16-HBE cell lines. The 584-mimic was used to increase the expression of miR-584, and the NC-mimic was used to add a negative control sequence. After 24h of transfection, the cells were incubated with LPS for another 24h, and the effects on the release of inflammatory cytokines, such as IL-1, IL-6, IL-8, TNF-α, MIP-1, MCP-1α, and cell viability were investigated. The optimal conditions for transfection were evaluated, and cytokine and chemokine mRNA levels were determined. Regardless of the NC- or 584-mimic, the results indicate that the expression of transfected genes in these cells leads to an increase in inflammatory factors and decreased cell viability. Microscope analysis revealed that the number of HBE cells was lower after transfection, and many small vesicles could be observed in the transfected cells, indicating that the insertion of gene vectors may affect the biological activity of HBE cells and experimental results. Results suggest that Lipofectamine 3000 transfected miRNA into HBE cells, providing better transfection rates, however, at the cost of higher toxicity.

Key words: Cell transfection, Lipofectamine 3000, HBE cells, Inflammatory factors, miR-584-mimic

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Introduction

Transfection is an experimental technique typically used in biological experiments (Zhou et al., 2013). Cell transfection involves introducing foreign nucleic acids, DNA or RNA, into eukaryotic cells to achieve genetic mutation or lowering protein levels by RNAi and can be classified as physically, chemically, or biologically mediated (Zolfaghari et al., 2020). Liposomal transfection belongs to the chemically mediated class, presents a high transfection rate, is suitable for transfecting DNA into suspensions or adherent cultured cells, and is one of the most convenient transfection methods in the laboratory (Zhang and Li, 2020). Many factors affect the outcome of cell transfection, such as the growth state of the cells (Zwick et al., 2016), and neither primary cells nor cells with many passages are ideal for achieving successful transfection (Tamai, 1996). Cells in the logarithmic growth phase reportedly have the highest growth and are the most suitable for transfection (Singh et al., 2013). Other factors to consider are a cell density of about 30% to 40% confluency before transfection, which should not be too high to avoid growing too full and causing cell death, and transfection time, which should not be too long, about 24 hours (Rashid and Coombs et al., 2019), as these factors cause the cells to detach and float to the surface when it is time to conduct the experiments. Therefore, the cell growth cycle and time of transfection should be considered before plating (Zuo et al., 2012; Zwick et al., 2016). Human bronchial epithelial (16-HBE) cells are adherent cells that grow rapidly and are easy to transfect (Wang et al., 2016). Among the methods for liposomal transfection, we selected Lipofectamine 3000 to transfect microRNA (miRNA) miR-584-mimic in 16-HBE cells. When studying the effect of transfection of the miR-584-mimic gene on lipopolysaccharide (LPS)-induced inflammation in bronchial epithelial cells, whether the biological changes caused by gene silencing or overexpression induced by transfection affect the experimental results should be clarified. These aspects have not been explored before;

hence our study aimed to elucidate whether transfection affects inflammatory cytokines and chemokines.

Materials and methods

Cell culture

The human bronchial epithelial (16-HBE) cell line was provided by the Basic Department of Chengde Medical College (Chengde, Hebei, China). Briefly, 16-HBE cells were incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum (Vivacell, Shanghai, China) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified 37 °C incubator with 5% CO_2 (Zou et al., 2021a). To determine the optimal density, three different concentration gradients were used in a six-well plate: 2×10^5, 4×10^5, and 8×10^5 cells/well. Finally, the 16-HBE cells with the determined optimal density of 4×10^5 cells/well were incubated for 24h.

Cell transfection

To achieve miR-584-5p overexpression in HBE cells, Lipofectamine 3000 (Zhao et al., 2021) was used to transfect 584-mimic and NC-mimic into HBE cells to target gene reconstruction. The negative control mimic (NC-mimic) and the miR-584-mimic (584-mimic) were purchased from Zhongshi Gene Technology Co. Ltd. (Tianjin, China) and transfected into HBE cell lines with Lipofectamine 3000 (Invitrogen), in accordance with the manufacturer's instructions. The 584-mimic was used to increase the expression of miR-584, and the NC-mimic was used to add a negative control sequence (Zuo et al., 2019a). After 24h of transfection, the cells were incubated with LPS (100 μg/mL, Sigma, St. Louis, MO, USA) for another 24h, and the effects on the release of inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-1(MIP-1), and monocyte chemoattractant protein-1α (MCP-1α), were investigated. According to the different transfectants and whether LPS was added, the cells were divided into the following groups: control, LPS, NC-mimic, 584-mimic, NC-mimic+LPS, and 584-mimic+LPS.

With this experiment, we wanted to investigate the effects of transfection on inflammatory factor production, so we considered non-transfected cells as the control group, and transfected cells as the experimental group. We compared inflammatory cytokine DNA, such as IL-1, IL-6, IL-8, TNF-α, MIP-1, and MCP-1α between non-transfected and transfected 16-HBE cells (Fig. 2).

RNA extraction and qRT-PCR

Real-time quantitative PCR (qRT-PCR) was performed to evaluate the expression of cytokines and chemokines in HBE cell lines, and total RNA was extracted from HBE cells using the Superbrilliant™ 6-min high-purity RNA extraction kit (Zhongshi Gene Technology Co. Ltd., Tianjin, China), in accordance with the manufacturer's instructions. The RNA was then reverse-transcribed into cDNA using the FastKing RT Reagent (TianGen, Beijing, China) followed by qRT-PCR using a fully automatic fluorescent PCR analyzer (Roche Molecular Systems, Branchburg, NJ, USA) with SYBR Green SuperReal PreMix Plus with the following primers (GeneCopoeia, Inc., Rockville, MD, USA) (Table 1). GAPDH was used as the calibrator sample. The ΔCt value obtained for GAPDH was subtracted from the ΔCt obtained for the experimental sample and ΔΔCt was calculated (ΔCt_{Test}-ΔCT_{GAPDH} = ΔΔCt for the experimental sample). The RNA expression levels were determined via the $2^{-\Delta\Delta Ct}$ method.

Cell viability assay

Cell viability was assessed using a CCK8 proliferation detection kit (ReportBio, Hebei, China) (Zuo et al., 2019b). Approximately 4×10^4 cells/well were seeded in 96-well plates. After transfection and LPS incubation, 20 μL CCK8 solution was added to each well and cultured for 1h. The absorbance of the reaction system was measured spectrophotometrically at 450 nm (Lu, 2017).

Determination of gene transfection efficacy

After transfection and LPS stimulation, total miRNA was extracted from HBE cells in accordance with the manufacturer's protocol using a Superbrilliant™ cell miRNA extraction kit (Zhongshi Gene Technology Co. Ltd). miRNA expression was analyzed using the miRNA reverse transcription reagent (Zhongshi Gene Technology Co. Ltd). qRT-PCR was performed using a fully automatic fluorescent PCR analyzer (Roche Molecular Systems) with the Supersmart™ TaqMan miRNA Quantitative PCR Probe for miR-584-5p and

Table 1. Gene name and primer sequences.

Gene	Primer sequence	
IL-1	Forward	5'-TGTATGTGACTGCCCAAGATGAAG-3'
	Reverse	5'-AGAGGAGGTTGGTCTCACTACC-3'
IL-6	Forward	5'-AGACAGCCACTCACCTCTTCAG-3'
	Reverse	5'-TTCTGCCAGTGCCTCTTTGCTG-3'
IL-8	Forward	5'-GAGAGTGATTGAGAGTGGACCAC-3'
	Reverse	5'-CACAACCCTCTGCACCCAGTTT-3'
TNF-α	Forward	5'-CTCTTCTGCCTGCTGCACTTTG-3'
	Reverse	5'-ATGGGCTACAGGCTTGTCACTC-3'
MCP-1	Forward	5'-AGAATCACCAGCAGCAAGTGTCC-3'
	Reverse	5'-TCCTGAACCCACTTCTGCTTGG-3'
MIP-1α	Forward	5'-ACTTTGAGACGAGCAGCCAGTG-3'
	Reverse	5'-TTTCTGGACCCACTCCTCACTG-3'
GAPDH	Forward	5'-GTCTCCTCTGACTTCAACAGCG-3'
	Reverse	5'-ACCACCCTGTTGCTGTAGCCAA-3'

RNU6 (internal group). miRNA expression levels were normalized to RNU6 via the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to compare the data using SPSS software version 26.0. The LSD method was used to compare indexes between groups, and statistical significance was set at $p<0.05$.

Results

Optimal conditions for transfection

This study comprehensively evaluated factors such as transfection reagent, gene sequence, and cell number, optimized the transfection of HBE cells, and provided a basis for cell transfection. Our results show that cell density is also important, recommending a 30% to 40% density range for cell transfection (Rashid and Coombs et al., 2019). However, we found that the optimal density was 80% before transfection. We found that a combination of 0.1 nmol gene sequence, 10 μL Lipofectamine 3000 transfection reagent, and 4×10^4 cells/well achieved the highest transfection rate.

Transfection efficiency of HBE cells and miR-584 gene expression

Nucleic acid verification is the most accurate method to establish the transfection efficiency (Zhou et al., 2016); in this study, qRT-PCR revealed that the expression of miR-584 RNA was 300-fold higher in HBE cells transfected with 584-mimic compared with HBE cells transfected with NC-mimic (Fig. 1).

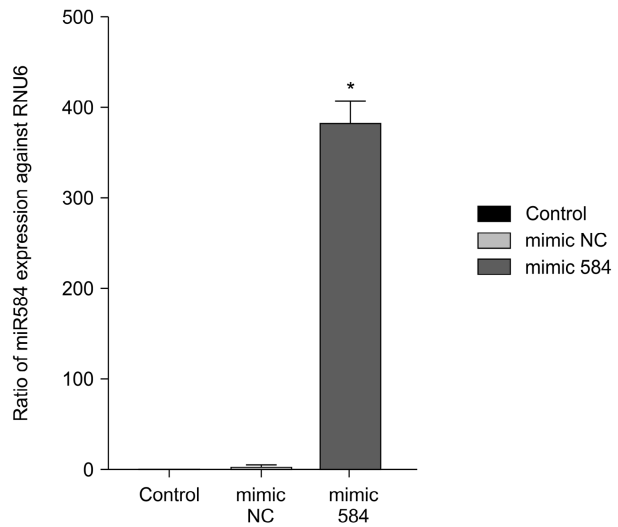

Fig. 1. miR-584 gene expression in 16-HBE cells 24h after transfection. Briefly, 16-HBE cells were transfected with a negative control (NC)-mimic or miR-584-mimic for 24h. Non- transfected HBE cells were used as a control. miR-584 gene expression was measured via qRT-PCR. *$p<0.05$ compared with the control and NC-mimic groups.

Cytokine and chemokine mRNA expression in HBE cells and morphological changes after transfection

A progressive increase in cytokine and chemokine mRNA expression was observed in HBE cells after transfection (Fig. 2). The number of cells was reduced and vesicles occurred after transfection with both miR-584-mimic and NC-mimic (Fig. 3)

Viability of HBE cells upon transfection

The CCK8 assay evaluated the relative cell viability of 16-HBE cells. Through CCK8 assays, we demonstrated that the viability of 16-HBE cells after transfection was decreased both with and without LPS (Fig. 4).

Discussion

TargetScan software showed that miR-584 can bind to the 3′ non-coding region of the *TLR* gene, thereby affecting the inflammatory pathway and the development of inflammation. This study aimed to explore the role of miR-584-5p in regulating the inflammatory reaction of HBE cells *in vitro*. In this study, we obtained transient-transfected 16-HBE cell lines with the commonly used Lipofectamine 3000 (Zou

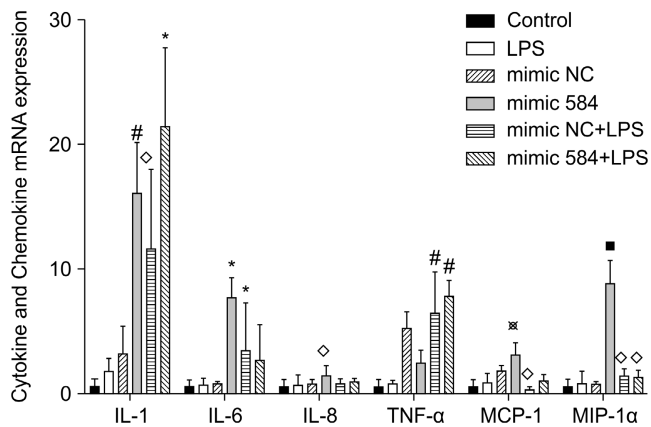

Fig. 2. Relative mRNA expression of cytokines and chemokines via real-time RT-qPCR in HBE cells transfected with negative control (NC)-mimic or miR-584-mimic. HBE cells were transfected with either NC-mimic or 584-mimic for 24h and then stimulated with LPS (100 μg/mL) for 24h. Non- transfected HBE cells were used as a control. Non-transfected HBE cells treated with lipopolysaccharides (LPS) represent the LPS group. The mRNA levels of IL-1, IL-6, IL-8, TNF-α, MCP-1, and MIP-1α in HBE cells were measured via RT-qPCR. GAPDH served as an internal control. *$p<0.05$ compared with the control, LPS, and NC-mimic groups; #$p<0.05$ compared with the control and LPS groups. ◇$p<0.05$ compared with the control group. ■$p<0.05$ compared with the control, LPS, NC-mimic, NC-mimic+LPS and 584-mimic+LPS groups. At least three independent experiments were performed. Results are presented as the mean ± SD.

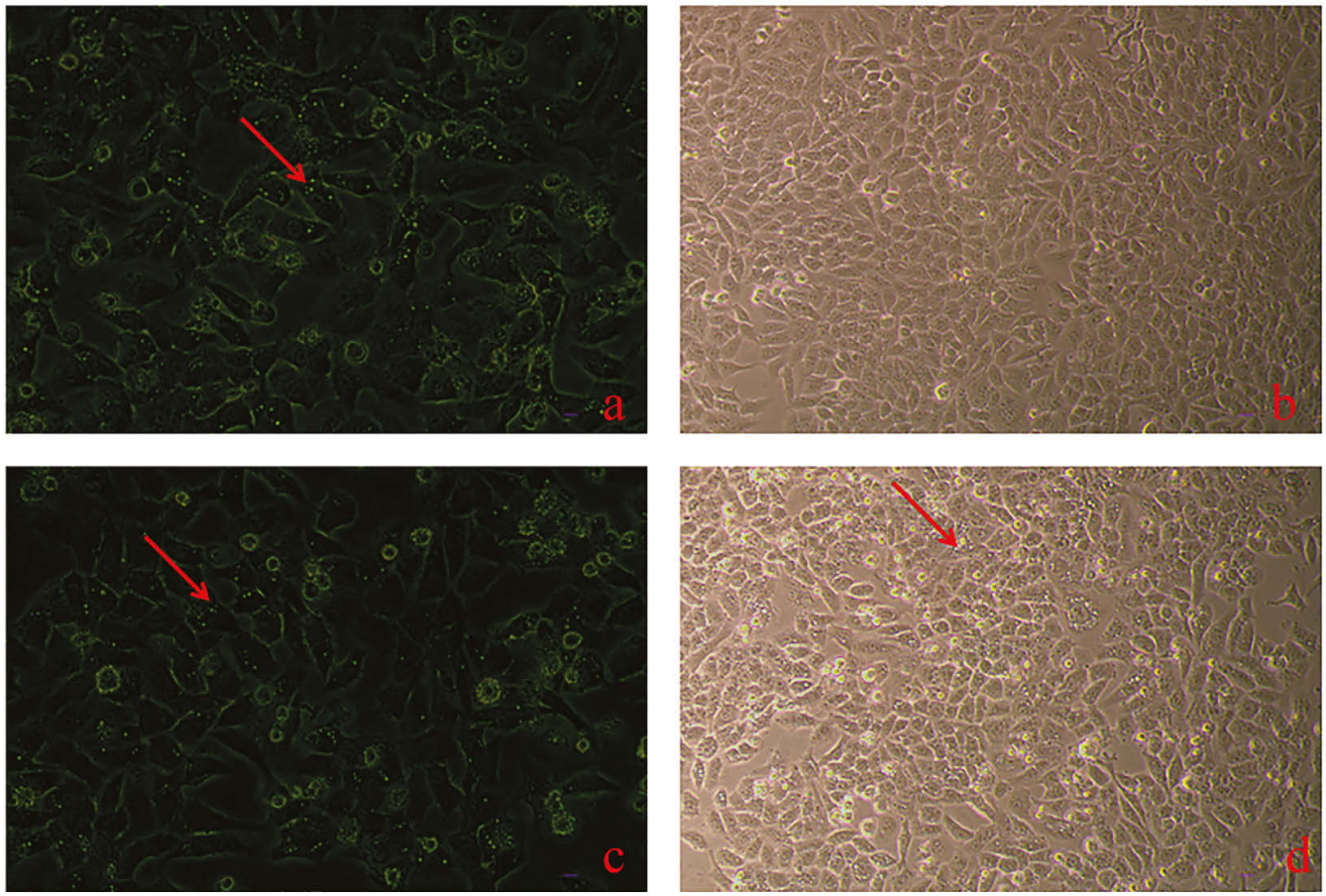

Fig. 3. Representative sections of HBE cells 24h after transfection **a, b.** HBE cells were transfected with NC-mimic. **c, d.** HBE cells were transfected with 584-mimic). The transfected cells were labeled with green fluorescent dye. Pictures were obtained via fluorescence microscopy. The representative sections are shown at 200× original magnification. The number of HBE cells decreased after transfection, the cells became round and many small vesicles can be observed in the transfected cells. Red arrows indicate the green fluorescent staining (left) and vesicles (right) in the transfected HBE cells.

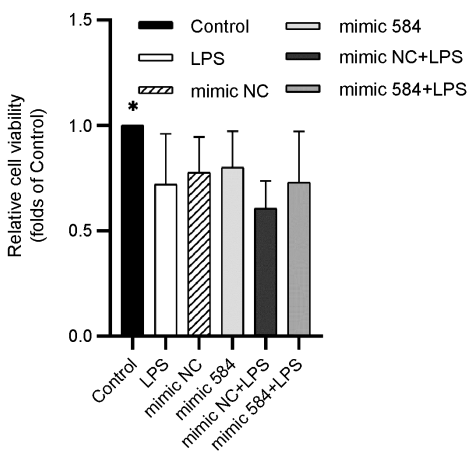

Fig. 4. CCK8 measurement of the relative cell viability of 16-HBE cells. Briefly, 16-HBE cells were transfected with NC-mimic or 584-mimic and then treated with LPS for the indicated time. *p<0.05 compared with the LPS, NC-mimic, 584-mimic, NC-mimic+LPS, and 584-mimic+LPS groups. At least three independent experiments were performed. Results are presented as the mean ± SD.

et al., 2021b). Lipofectamine 3000 has been used to deliver RNAs (Rust et al., 2015) into various cell lines and can be a highly efficient nonviral vector for various gene therapy applications (Wang et al., 2018). These results suggest that HBE cells may serve as vehicles for the *in vitro* introduction of functional recombinant genes.

Our results indicate that the expression of transfected genes in these cells leads to increased levels of inflammatory factors and affects cell viability, regardless of transfection with either the 584- or NC-mimic by Lipofectamine 3000. The interference of transfection with HBE cell division may be partly responsible for the release of cytokines and chemokines. The genes may be modified by the incorporation of mimics and negative controls through transfection, which can change cell gene expression and structure (Żydowicz-Machtel et al., 2021), and may increase the expression of inflammatory factors. The ability of Lipofectamine 3000 to transfect miRNA to target genes may change the level of inflammatory factors; small

vesicles were observed and the number of HBE cells was lower after transfection, which was confirmed by the decreased cell viability of the transfected cells. Thus, the insertion of gene vectors may affect the biological activity of HBE cells and the test results. According to the results in Figure 2, using lipofectamine 3000 may affect the experiment results, so it is important to choose the control group. The control group should be chosen from samples transfected with a negative control sequence instead of non-transfected samples, to balance the influence of lipofectamine 3000 transfection on the experimental result.

Our results suggest that transfection can regulate the expression of cytokines and chemokines, which may be related to phenotypic changes and decreased cell viability; however, the mechanism of transfection affecting inflammatory factors has not been studied and may involve multiple unidentified signaling pathways. When an experiment involves transfection, the effect of transfection on therapy should be considered. We need a new competitive vehicle for gene delivery applications; a method with fewer side effects that can be applied to cell transfection (Yu et al., 2017) should be established to facilitate the study of the therapeutic efficacy of gene overexpression and silencing.

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Author contributions. Bo Zhang designed and performed the experiments, analyzed the data, and wrote the first draft of the manuscript. Jie Cao designed and conducted the project.

Competing interests. The authors have no relevant financial or non-financial interests to disclose.

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