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# MiR-34 promotes apoptosis of lens epithelial cells in cataract rats *via* the TGF-β/Smads signaling pathway

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**Abstract. – OBJECTIVE: To discuss the effect of micro ribonucleic acid (miR)-34 on the lens epithelial cell functions in the cataract rats.**

**MATERIALS AND METHODS: Differentially expressed miRNAs in the lens epithelial cells of the cataract rats were screened out by analyzing microarrays. The lens epithelial cells of the cataract rats transfected with miR-34 mimics were set as transfection group. Cells with silenced transforming growth factor-β (TGF-β) using RepSox were regarded as the transfection + inhibitor group, and the cells transfected with NC constituted control group. Relative expressions of miR-34, key genes in the TGF-β/Smads signaling pathway and apoptosis-related proteins [B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), Bcl-2 associated K protein (Bak), caspase-9 and surviving] in the control group, transfection group, and transfection + inhibitor group were detected. The proportions of apoptotic cells in the three groups were determined *via* flow cytometry.**

**RESULTS: The differentially expressed miRNAs in the lens epithelial cells of the cataract rats included miR-5, miR-128, etc. Among the tested miRNAs, miR-34 presented remarkably downregulated expression [log2 fold change (FC) =-2.11, *p*=0.000]. After the lens epithelial cells of the cataract rats were transfected with miR-34 mimics, the expression of miR-34 was evidently elevated (p=0.000), while the expressions of TGF-β, Smad1, and Smad3 were significantly up-regulated. Following the treatment with the TGF-β inhibitor RepSox, the expressions of TGF-β, Smad1, and Smad3 were downregulated. After transfection of miR-34 mimics in lens epithelial cells of the cataract rats, upregulated Bax and Bak, downregulated Bcl-2 and surviving, and elevated apoptosis rate were observed. After the TGF-β inhibitor RepSox was added, the expressions of Bax and Bak declined prominently, while those of Bcl-2 and survivin were on the contrary, manifesting a declining cell apoptosis rate. The expression of caspase-9 had no significant change among the three groups. The proportion of apoptotic cells in control group, transfection group, and transfection + inhibitor group was 2.33%, 38.14%, and 16.88%, respectively, displaying differences among the three groups (*p*=0.002).**

**CONCLUSIONS: MiR-34 can promote the apoptosis in lens epithelial cells of cataract rats *via* the TGF-β/Smads signaling pathway.**

*Key Words:*
MiR-34, Lens epithelial cells, TGF-β/Smads signaling pathway, Cataract.

## Introduction

Cataract has become the principal disease affecting the visual sense of the middle-aged and senior people, posing a great impact on their production activities[1,2]. Operation is generally preferred to cataract. However, after-cataract is common after surgical procedures, with the incidence of 20-80%. Recent studies[3] have shown that after-cataract is triggered by the too fast growth of lens epithelial cells that go beyond the posterior capsule. Functional changes of lens epithelial cell functions are vital for postoperative complications in cataract patients. The enhanced proliferative function of the lens epithelial cells will aggravate disease severity of cataract and reduce the surgical curative effect. On the contrary, improved apoptosis of lens epithelial cells mitigates the illness[4]. Various cytokines and proteins can facilitate cell apoptosis. Exploring the factors stimulating the lens epithelial cell apoptosis is conducive to the treatment of postoperative complications for cataract patients.

Micro ribonucleic acids (miRNAs) are non-coding RNAs obtained through the genomic deoxyribonucleic acid (DNA) transcription[5]. It was previously considered that MiRNAs could not

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encode and synthesize proteins, so they might not have too much effect. Nevertheless, miRNAs can exert crucial regulatory effects on normal physiological activities or tumor processes of human beings[6,7]. MiRNAs can modulate the intracellular transcription process and influence the protein expressions, so as to affect the transduction of the signaling pathway and the intensity of substance metabolism by binding to messenger RNAs (mRNAs). MiR-184 is identified to be the indicator for the recurrence of women's low-risk endometrial cancer[8]. The expressions of miR-208a, miR-208b, and miR-499 are correlated with myocardial damage of children after cardiac surgery[9]. Furthermore, the expression profile of miRNAs is related to the occurrence of cervical cancer[10]. MiR-34 may play a critical role in regulating the development of cataract. The transforming growth factor-β (TGF-β)/Smads signaling pathway participates in the disease progression[11].

In this study, the effects of miR-34 on apoptosis of lens epithelial cells extracted from cataract rats and the involvement of the TGF-β/Smads signaling pathway were detected.

## Materials and Methods

### Materials

Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA), fetal bovine serum (FBS; Gibco, Rockville, MD, USA), trypsin, phosphate-buffered saline (PBS) (powder), TGF-β inhibitor RepSox (Selleck, Houston, TX, USA), miR-34 mimics, mRNA primers of miR-34, TGF-β, Smad1, Smad3, B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), Bcl-2 associated K protein (Bak), caspase-9 and survivin (synthesized by Shanghai Sangon, Shanghai, China), polyclonal antibodies of TGF-β, Smad1 and Smad3 (Abcam, Cambridge, MA, USA), and Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI; Shanghai Rainbow, Shanghai, China).

### Detection of Differentially Expressed MiRNAs in Lens Epithelial Cells of Cataract Rats

Detection of differentially expressed miRNAs between the lens epithelial cells and normal epithelial cells of the cataract rats was authorized by Shanghai Sangon Biotech by analyzing Affymetrix microarrays (Santa Clara, CA, USA).

### Transfection With miR-34 Mimics and Inhibition of TGF-β Expression by RepSox

The lens epithelial cells in the logarithmic growth phase were collected from the cataract rats. After one-day cell culture, serum-free Opti-minimal essential medium (MEM) was replaced. 5 μL of miR-34 mimics and 5 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were added with 245 μL of Opti-MEM, respectively. 5 min later, the two mixtures were gently mixed, let stand for 20 min, and placed into the cell culture medium. 2 μL TGF-β inhibitor RepSox was added 24 h later per well, followed by observation of the cell growth.

Grouping: the lens epithelial cells transfected with NC in the cataract rats were taken as the control group. Cells transfected with miR-34 mimics as the transfection group and those transfected with miR-34 mimics and TGF-β inhibitor RepSox as the transfection + inhibitor group.

### MRNA Detection

The mRNA expression levels of miR-34, TGF-β, Smad1, Smad3, Bax, Bak, caspase-9, Bcl-2, and survivin were detected *via* real-time quantitative polymerase chain reaction (qPCR), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference. Total RNAs in the cells were extracted using TRIzol reagent method (Invitrogen, Carlsbad, CA, USA). 1 μL of RNA sample was taken and diluted at 1:50, and $OD_{260}/OD_{280}$ ratio was detected. Qualified RNAs ($OD_{260}/OD_{280}$ >1.8) were reversely transcribed into complementary deoxyribonucleic acids (cDNAs), and preserved at –20°C. The gene-specific primers were designed *via* Primer Premier 5.0 and synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Primer sequences and temperature (Tm) values were seen in Table I. The total PCR system was of 25 μL, consisting of 1 μL of each primer, 0.5 μL of template cDNA, 12.5 μL of SYBR Premix Taq and 10 μL of $dH_2O$. The PCR conditions: 95°C for 3 min, followed by 45 cycles at 95°C for 30 s, 59°C for 45 s and 72°C for 35 s.

### Western Blotting

Protein expressions of TGF-β, Smad1, and Smad3 were detected *via* Western blotting, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference. Cells were lysed in radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China) containing 1% phenylmethylsulfonyl fluoride (PMSF) protease inhibitor on ice for 30 min, during which

**Table I.** QPCR primer sequences.

| Gene | | Primer sequence 5′→3′ | Tm |
|---|---|---|---|
| MiR-34 | Forward | GAGGCCAAGCCCTGGTATG | 60.5 |
| | Reverse | CGGGCCGATTGATCTCAGC | 60.6 |
| TGF-β | Forward | CCTCTCTCTAATCAGCCCTCTG | 60 |
| | Reverse | GAGGACCTGGGAGTAGATGAG | 61.2 |
| Smad1 | Forward | CTGAACTTCGGGGTGATCGG | 61.6 |
| | Reverse | GGCTTGTCACTCGAATTTTGAGA | 62.5 |
| Smad3 | Forward | CCTGTAGCCCACGTCGTAG | 61 |
| | Reverse | GGGAGTAGACAAGGTACAACCC | 60.7 |
| Bax | Forward | CCCTCACACTCAGATCATCTTCT | 61.1 |
| | Reverse | GCTACGACGTGGGCTACAG | 61.7 |
| Caspase-9 | Forward | AAAATGTCCCTCCGTTCTTATGG | 60.5 |
| | Reverse | CTGAAGTTGAGCGTAATACCAGT | 60.6 |
| Bcl-2 | Forward | GACGTGGAACTGGCAGAAGAG | 60.5 |
| | Reverse | TTGGTGGTTTGTGAGTGTGAG | 60.7 |
| Survivin | Forward | AGACAGGGGCCTTTTTGCTAC | 62.4 |
| | Reverse | AATTCGCCGGAGACACTCG | 60.6 |
| Bak | Forward | CCCGAGAGGTCTTTTTCCGAG | 61.2 |
| | Reverse | CCAGCCCATGATGGTTCTGAT | 60.8 |
| GAPDH | Forward | GGAGCGAGATCCCTCCAAAAT | 62.3 |
| | Reverse | GGCTGTTGTCATACTTCTCATGG | 62.3 |
| U6 | Forward | GCGCGTCGTGAAGCGTTC | 60.5 |
| | Reverse | GTGCAGGGTCCGAGGT | 60.5 |

vortex mixing was performed for 3 times. The cells were centrifuged at 14,000 rpm for 14 min, bathed in boiling water after the loading buffer was added, and then preserved at –20°C. Subsequently, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for 1 h. Protein samples were transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and then blocked in 5% skim milk. Subsequently, membranes were incubated with the antibodies at 4°C overnight. After rinsing the next day, the horseradish peroxidase (HRP)-labeled secondary antibodies were added and incubated for 1 h, washed and then exposed for bands.

### Proportion of Apoptotic Cells Detected Via Flow Cytometry

The lens epithelial cells of the cataract rats in the control group, transfection group, and transfection + inhibitor group were stained using Annexin V-FITC and PI. Absorbances at 488 nm (excitation wavelength), 515 nm (FITC wavelength) and 560 nm (PI wavelength) were recorded.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. Student's *t*-test was used for comparison of two groups, and comparison between groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $p<0.05$ indicated statistical significance.

## Results

### Differentially Expressed miRNAs in Lens Epithelial Cells of Cataract Rats

The differential expression profiles of miRNAs in the lens epithelial cells of the cataract rats were seen in Figure 1 and Figure 2. According to Figure 1, miR-34 was lowly expressed in the lens epithelial cells of the cataract rats [$\log_2$ fold change (FC) =-2.11, $p$=0.000], indicating that miR-34 may exert vital functions in them. Some miRNAs such as miR-5 and miR-128 that were differentially expressed in the lens epithelial cells of the cataract rats were classified into the same category (Figure 2).

### MiR-34 Expressions in Control Group and Transfection Group

The expression levels of miR-34 in the control group and transfection group were shown in Figure 3. After the lens epithelial cells of the cat-

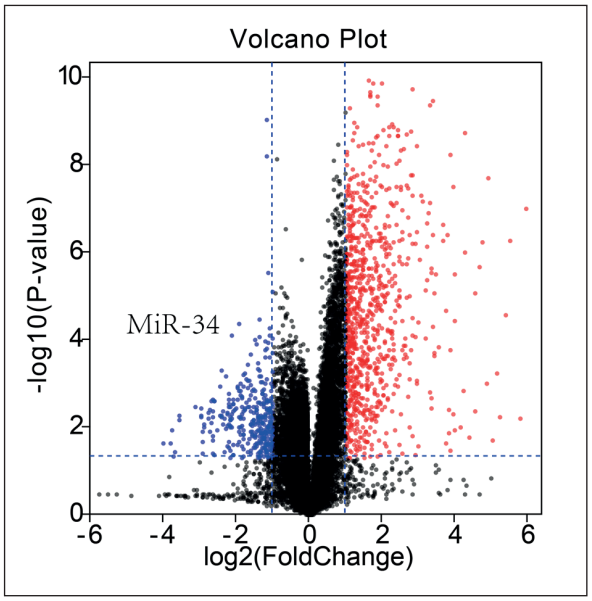


**Figure 1.** Volcano plots of differentially expressed miRNAs in lens epithelial cells of cataract rats. Each plot in the figure represents a miRNA in the lens epithelial cells of the cataract rats. Red plots are up-regulated miRNAs with $\log_2$FC >1 and $p$<0.05, and blue ones are down-regulated miRNAs with $\log_2$FC <-1 and $p$<0.05.

aract rats in transfection group were transfected with miR-34 mimics, the miR-34 expression was markedly elevated ($p$=0.000).

### *MRNA and Protein Expressions of genes in TGF-β/Smads Signaling Pathway in Control Group, Transfection Group, and Transfection + Inhibitor Group*

The mRNA expressions of key genes in the TGF-β/Smads signaling pathway in control group, transfection group, and transfection+inhibitor group were displayed in Figure 4. After the lens epithelial cells from the cataract rats were transfected with miR-34 mimics, the expressions of TGF-β, Smad1, and Smad3 were remarkably upregulated. However, their increased levels were significantly reduced following the treatment with the TGF-β inhibitor RepSox. As shown in Figure 5, the protein expressions of TGF-β, Smad1, and Smad3 in the control group, transfection group, and transfection + inhibitor group were identical with the tendency of mRNA expressions.

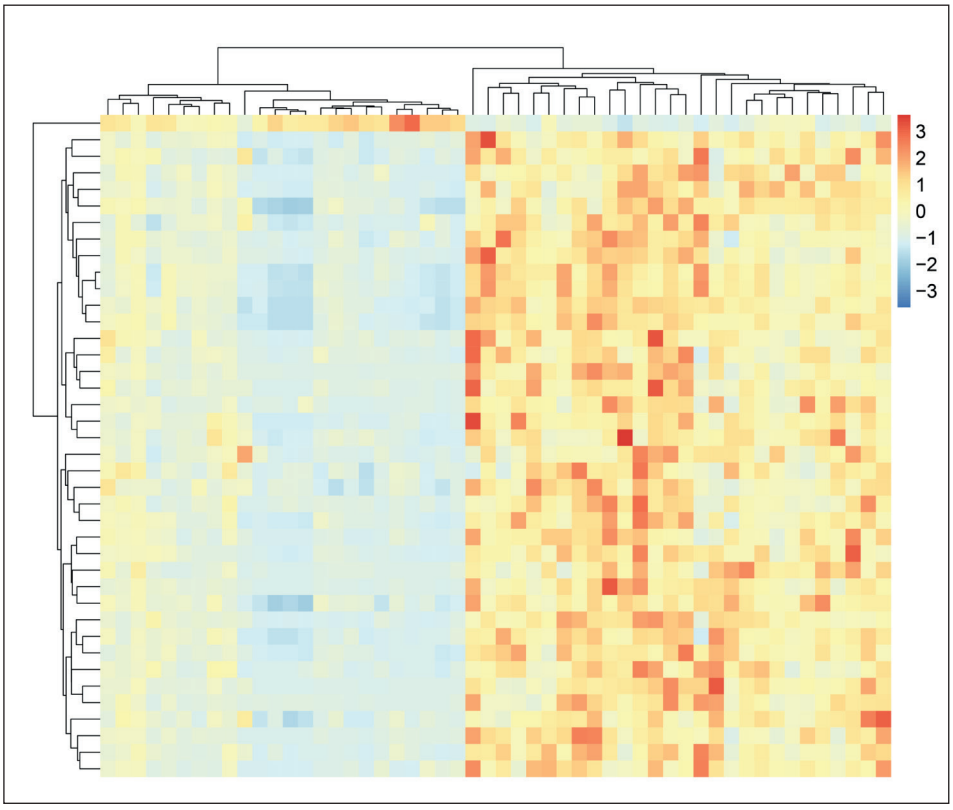


**Figure 2.** Heat map of differentially expressed miRNAs in lens epithelial cells of cataract rats. The row in the figure represents lens epithelial cell specimens and normal cell specimens from the cataract rats, and the column represents miRNAs with significantly differential expressions ($p$<0.05). The color shade indicates the expression level of miRNAs.

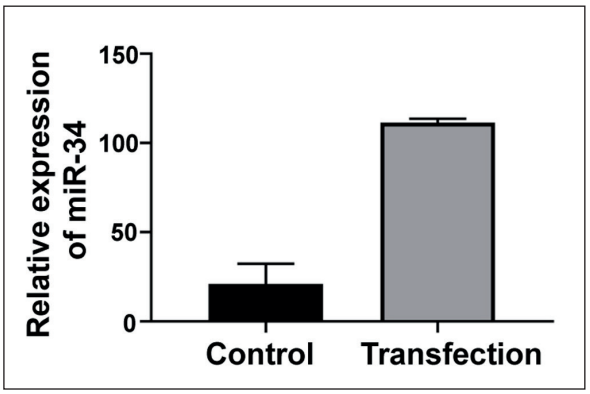


**Figure 3.** MiR-34 expressions in control group and transfection group. The relative expression of miR-34 in the control group and transfection group is (25.11±4.23) and (123.24±3.14), respectively. The difference between the two groups is of statistical significance ($p$<0.05).

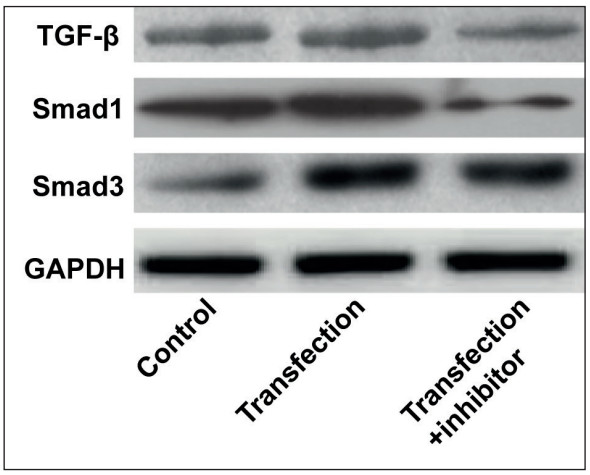


**Figure 5.** Protein expressions of key genes in the TGF-β/Smads signaling pathway in control group, transfection group and transfection + inhibitor group.

### *Expressions of Apoptosis-Related Proteins in Control Group, Transfection Group, and Transfection + Inhibitor Group*

The expression levels of apoptosis-related proteins in the control group, transfection group, and transfection + inhibitor group were exhibited in Figure 6. After the lens epithelial cells of the cataract rats were transfected with miR-34 mimics, the expression levels of Bax and Bak notably increased, while those of Bcl-2 and survivin were evidently reduced, and the cell apoptosis was enhanced. After the cells were treated with the TGF-β inhibitor RepSox, the expressions of Bax and Bak declined prominently, while those of Bcl-2 and survivin increased distinctly, manifesting the reduced cell apoptosis. The expression of caspase-9 had no significant change.

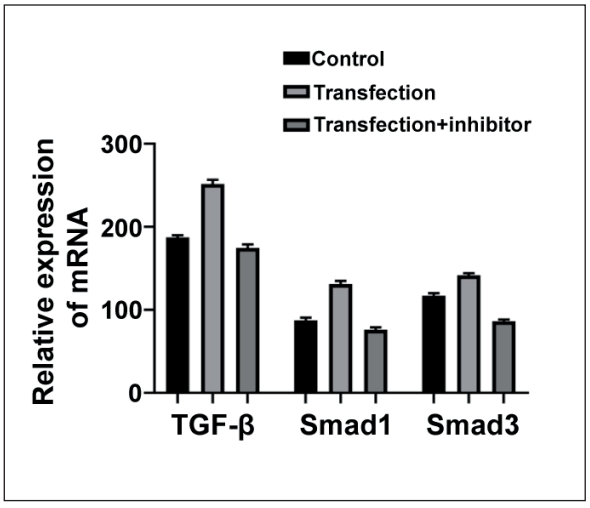


**Figure 4.** MRNA expressions of key genes in the TGF-β/Smads signaling pathway in control group, transfection group and transfection + inhibitor group. Differences in the expressions of TGF-β, Smad1 and Smad3 among the control group, transfection group and transfection + inhibitor group have statistical significance ($p$<0.05).

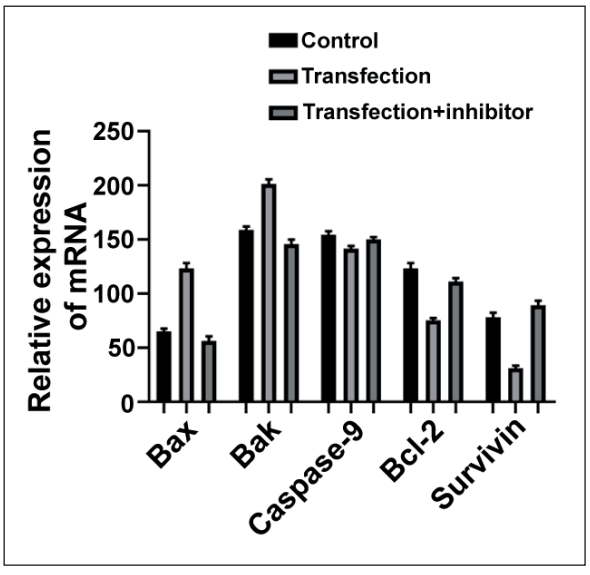


**Figure 6.** Changes in apoptotic protein levels in control group, transfection group and transfection + inhibitor group. Control group, transfection group and transfection + inhibitor group are statistically different in the expression levels of Bax, Bak, Bcl-2 and survivin ($p$<0.05) but not statistically different in the caspase-9 expression ($p$=0.216).

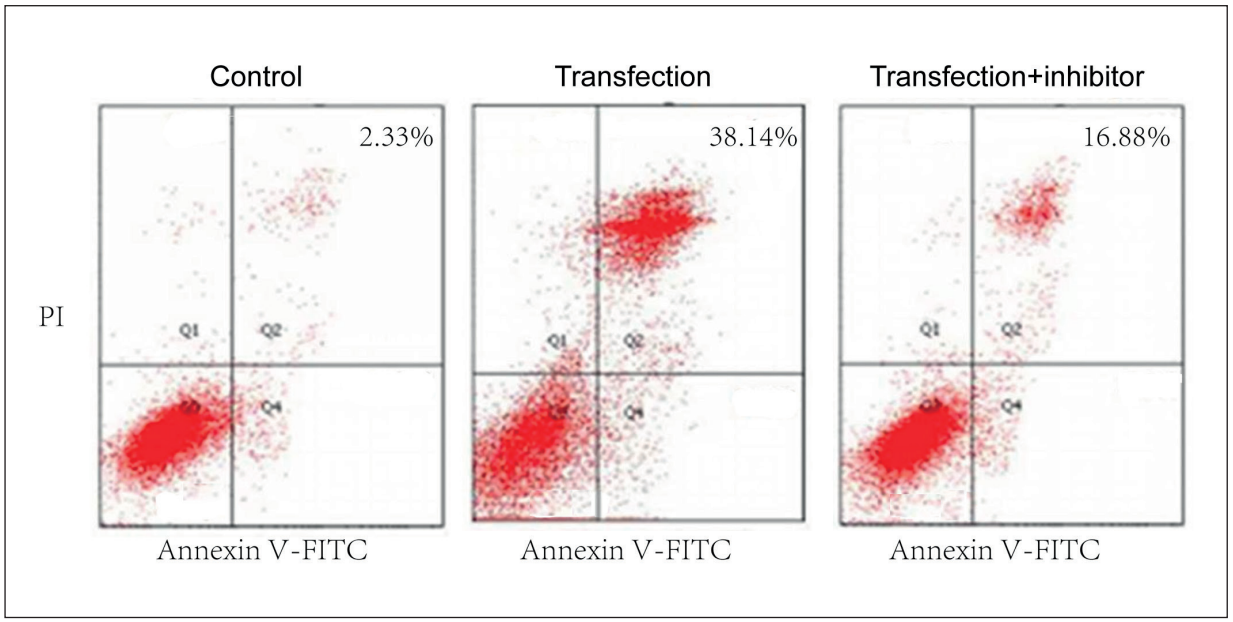


**Figure 7.** Proportions of apoptotic cells in control group, transfection group and transfection + inhibitor group. Percentage in the figure stands for the proportion of apoptotic cells in each group. Differences in the proportion of apoptotic cells among the control group, transfection group and transfection + inhibitor group are statistically significant ($p<0.05$).

### *Comparison of Proportions of Apoptotic Cells Among Control Group, Transfection Group, and Transfection + Inhibitor Group*

The proportions of apoptotic cells in the control group, transfection group, and transfection + inhibitor group were seen in Figure 7. The proportion of apoptotic cells in the control group, transfection group, and transfection + inhibitor group was 2.33%, 38.14% and 16.88%, respectively, with statistically significant differences among the three groups ($p=0.002$).

## Discussion

The primary postoperative complication of cataract is after-cataract involving the lens epithelial cell functions[12]. The proliferation of the lens epithelial cells aggravates complications of cataract. Cell death can be divided into pyroptosis, autophagy, and apoptosis, and the latter is the most common one. The cell apoptosis process is mainly regulated by caspases and many other factors. Transcription and translation of pro-apoptotic proteins significantly influence cell apoptosis. Therefore, studying the factors stimulating the lens epithelial cell apoptosis will be considerably significant for reducing the occurrence of postoperative complications of cataract.

As a vital constituent part of transcription products of non-coding genes, miRNAs are able to regulate expressions of target genes. According to this study, a large number of miRNAs such as miR-5 and miR-128 were differentially expressed in the lens epithelial cells of the cataract rats. They may be able to affect functions of lens epithelial cells, such as EMT, cell growth, and migration. It has been affirmed that some miRNAs have the abilities to affect the progression of diseases or tumors, including malignant glioma[13], lung cancer[14], breast cancer[15], and Alzheimer's disease[16]. MiR-34 has been found to act on diversified diseases. As a therapeutic target of some tumors[17], it can exert an effect on the tumor cell apoptosis[18] and correlated with virus infection[19].

It was discovered in this study that miR-34 presented significantly low expression in the lens epithelial cells of the cataract rats ($\log_2 FC=-2.11$, $p=0.000$). As miRNAs are able to modulate the expressions of some pathway-related genes after binding to coding genes[20], whether the expressions of related proteins in the TGF-β/Smads signaling pathway were influenced by miR-34 was detected. After overexpression of miR-34 in lens epithelial cells, the expressions of TGF-β, Smad1, and Smad3

in the TGF-β/Smads signaling pathway were significantly up-regulated. After the treatment with the TGF-β inhibitor RepSox, the expressions of TGF-β, Smad1, and Smad3 markedly dropped and the TGF-β/Smads pathway was inactivated. As shown in Figure 5, the protein expressions of TGF-β, Smad1 and Smad3 in the control group, transfection group, and transfection + inhibitor group presented the same tendency as the mRNA expressions. Hence, miR-34 can activate the TGF-β/Smads pathway to further regulate the functions and differentiation of lens epithelial cells in the cataract rats.

Subsequently, it was certified that miR-34 posed an evident impact on apoptosis of the lens epithelial cells of the cataract rats. After transfection of miR-34 mimics, apoptosis rate in lens epithelial cells of the cataract rats was enhanced (38.14%). Meanwhile, expressions of pro-apoptotic proteins Bax and Bak were evidently up-regulated, while those of anti-apoptotic proteins Bcl-2 and survivin significantly declined. TGF-β inhibitor RepSox markedly reversed the above trends. The expression of pro-apoptotic protein caspase-9 was not altered by miR-34 ($p$=0.216) and TGF-β inhibitor RepSox ($p$=0.081), manifesting that miR-34 cannot facilitate the lens epithelial cell apoptosis in the cataract rats by influencing caspase-9.

## Conclusions

In summary, we indicated that miR-34 can promote the lens epithelial cell apoptosis in cataract rats *via* the TGF-β/Smads signaling pathway.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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