· Original article ·

Effect of integrin-linked kinase on the proliferation and migration of human retinal pigment epithelium cells

Yi-Xia Zhang[1], *Xiao-Yang Zhang*[2], *Ning-Ning Chen*[1], *Cai-Yun Li*[1]

[1]Department of Ophthalmology, the First Affiliated Hospital of Shihezi University Medical College, Shihezi 832008, Xinjiang Uygur Autonomous Region, China
[2]Department of Geriatrics, the Fifth Affiliated Hospital, Xinjiang Medical University, Urumchi 830000, Xinjiang Uygur Autonomous Region, China
Correspondence to: Yi - Xia Zhang. Department of Ophthalmology, the First Affiliated Hospital of Shihezi University Medical College, Shihezi 832008, Xinjiang Uygur Autonomous Region, China. xyz991128@163. com
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Abstract

• AIM: To investigate the effect of RNA interference targeting integrin-linked kinase (ILK) on the proliferation and migration of cultured human retinal pigment epithelium (hRPE) cells.

• METHODS: hRPE cells were cultured, and their morphology and staining features were determined. ILK specific siRNA was designed and synthesized. siRNA was transfected into hRPE cells with Lipofectamine 2000. The effects of transfection were observed using a fluorescence microscope. RT - PCR and Western blot were used to semiquantitatively evaluate the mRNA and protein expressions of ILK. MTT assay was used to detect the proliferation of hRPE cells. Wound healing assay and transwell migration assay were used to detect the migration capability of hRPE cells.

• RESULTS: ILK was found in normal culture hRPE cells. The mRNA and protein expressions of ILK significantly decreased by siRNA transfection ($P<0.01$). The proliferation of hRPE cells also significantly decreased ($P<0.05$). The migration capability of hRPE significantly decreased by siRNA transfection, with statistically significant difference compared with the normal control group ($P<0.05$).

• CONCLUSION: ILK - siRNA transfection significantly decreases the mRNA and protein expressions of ILK, as well as the proliferation and migration abilities of hRPE cell's.

• KEYWORDS: RNA interference; ILK; retinal pigment epithelium; migration; proliferation

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INTRODUCTION

Retinal pigment epithelium (RPE) cells cause proliferative vitreoretinopathy (PVR) when they migrate to the vitreous cavity. They are also a major reason for retinal surgery failure. PVR has significant effects on visual function and is characterized by the contractility of the membrane in the vitreous and retinal surfaces containing RPE cells and proliferating fibroblast cells. The RPE cell is an important cellular component that does not proliferate under normal circumstances. Thus, the mechanism through which RPE cells leave their normal position in the vitreous cavity and begin abnormal proliferation remains unknown. This study investigates RNA interference silencing of ILK expression to verify its effects on RPE cell proliferation and migration.

MATERIALS AND METHODS

Materials The DMEM/F12 cell culture medium, optimum-minimum essential medium (Opti - MEM) serum - free medium, fetal bovine serum (Gibico Company, USA), Trizol reagent, a reverse transcription kit, and TAG enzymes were purchased from Beijing Zhong Shan Company (China). The protein extraction kit was purchased from Game Company. Standard protein marker was purchased from Sigma (USA). PCR primers were synthesized by the Shanghai GeneCore Biotech Company. Lipofectamine 2000 and siRNA were synthesized by Guangzhou Rainbow Biotechnology. A DAB kit, horseradish peroxidase-conjugated goat anti-rabbit IgG, quantitative PCR instrument, and other reagents used in the experiments were purchased from Beijing Zhong Shan Company (China).

Methods

Human RPE cells culture and identification Isolated and cultured corneal transplated donor eyes were used within 6 hours after death. The samples were gradually expanded by conventional culture passage with 5×10^7/L cell density, and inoculated in 6-well culture plates. Cells were identified by anti-human keratin antibody staining, which showed a purity of up to 100% of the RPE cells (Figure 1). Three to six cells were used in the experiments.

SiRNA synthesis The siRNA interference GAA-UCA-CUC-UGG-AGA-GCU-A dTdT ,dTdT CUU-AGU-GAG-ACC-UCU-CGA U(5'-3') was designed and synthesized according to the full-length human ILK gene sequence provided by GenBank.

siRNA transfection Trypsin-digested cells in the logarithmic phase were diluted with fresh medium without antibiotics

DMEM/F12 cells were passaged in 6-well plates, after antibiotic-free culture for 24 hours. At 60% cell fusion, transfection was begun. The culture medium was diluted with Opti-MEMI siRNA and transfection reagent at 50nmoL/L for transfection, following the operating instructions of the Lipofectamine 2000 reagents. The positive control transfection group was obtained by addition of si-β-actin to the medium, whereas the negative control group was obtain by adding an independent interference sequence. The simple liposome group did not have any siRNA. Six hours after transfection, the cells were cultivated and kept in continuous routine passages.

RT-PCR ILK primers 5'-GGCTCAGGATTTTCTCGCA -3', 5'-CTGCTGAGCGTCTGTTTGTG-3', which is 399bp, and an internal reference β-actin 5'-TGGATGATGATATCGCCGC-3', 5'-GTAGATGGGCACAGTGTGGGT-3', which is 501bp were used in study. We used 25μL of the primers during PCR to amplify the reaction. The product of the 1.5% agarose gel (containing 0.5g/L ethidium bromide) was photographed using camera. We also used Gel-Pro Analyzer 4.0 to determine the internal reference gene β-actin integral absorbance (*A*). We calculated the ratio of each band and the internal reference and considered, the relative *A* as the relative gene expression.

Western blot immunoblotting Based on the collected cell groups, we extracted total cellular proteins through Bradford determination of protein concentrations in the sample. Polyacrylamide glue was produced (12%, 5%). Glue was accumulated in the holes by addition of the same amount of sample and protein markers during the electrophoretic and transmembrane processes. We blocked the membranes with TBST (5% skim milk 10mmoL/L, Tris 100mmoL/L, NaCl, 0.1% Tween20, pH7.5) 1 hour at 37℃, incubated them overnight with 1:500 primary antibody (rabbit anti-ILK) at 4℃, and then washed them in TBST the next day. Horseradish peroxidase-conjugated secondary anti-IgG (1:800 dilution) was dropped into the membrane solution, which was shaken for 2 hours at room temperature, and then washed with TBST again. Finally, the membrane was incubated with ECL following the instructions that came with the reagent. Afterwards, the membrane was exposed to room temperature, Photographed, and analyzed for its protein bands A.

Thiazolyl blue (MTT) detection of hRPE cell proliferation The digested hRPE cells were inoculated in 96-well plates at a density of 1.5×10^5/mL. In each well, 200μL of 10% FBS DMEM/F12 medium was added 5% CO_2. The wells were then incubated at 37℃ for 24 hours. The cultured cells were transfected following the former treatment. Up to 50nmol/L siRNA-ILK was transfected into each cell group, after which continuous cultured continuously for 6 hours. 5g/L MTT at 50μL/well were added to the transfected cells, which were then incubated for 4 hours after continuous culturing at 12, 24, and 48 hours. We then added a triple solution composed of 10% sodium dodecyl sulfate-5% isobutanol -0.012mol/L hydrochloric acid (w/v/v) at 100μL/well. The solution was shaken for 10 minutes and then the *A* at 490nm was detected. Each group had six holes. The cell proliferation rate (%) was calculated as: = (OD value of experimental group-control OD value)/control OD value ×100.

Wound-healing experiments The normal culture group and transfection group were fused in monolayer using, 1% FBS 24 hours after curettaged cells with a 100μL pipette tip of 300μm to 500μm wide band. The cells were gently washed two times with PBS to remove non-adherent cells. RPE cells were cultured in 1% FBS continuously. Cellular morphology and migration were observed on two sides of the cell-free zone using a low power lens. The cell number in the cell-free zone was counted using a mesh counter equipped with a high-power lens. The migration ability of the cells was evaluated at 12 and 24 hours. Cell migration ability was calculated as = (migrating cell count of control group-migrating cell count of experimental group)/migrating cell count of control group × 100. The control group registered 0 percent cell migration ability.

Transwell cell migration assay We chose a 24-well plate to which, we added a 650μL of medium containing 10% fetal bovine serum as the chemotactic factor. In the transwell chamber leading into the hole, we selected two groups of hRPE cells for digestion. We prepared the cell suspension, and then trickled it into the end of the transwell chamber. The amount of indoor suspension was 100μL, the serum concentration was 1%, and the cell volume was 1×10^5/ holes. Eight hours later, we took out the transwell chamber, washed the cells with PBS 2 or 3 times, and then used a cotton swab to scrape the cells in the upper chamber. The lower cells were treated with Wright-Giemsa dye stain for 1 minute, washed with PBS, and then counted under a microscope.

Statistical Analysis The measured data were expressed as $\bar{x}\pm s$. We used ANOVA and independent samples *t*-test for determining the existence of significant difference. Statistical computations were conducted using SPSS 13.0 for Windows. $P<0.05$ was considered statistically significant.

RESULTS

Observed Under a Fluorescence Microscope Up to 50nmol/L of siRNA-ILK could be successfully transfected into hRPE cells, with a transfection efficiency of 72% (Figure 1).

RT-PCR and Western Blot Results In the positive control group, β-actin gene expression was significantly inhibited, showing a normal transfection system. Twenty-four hours after transfection of hRPE cells in ILK, mRNA relative expression levels in the normal culture group, liposome-alone group, negative control group and transfected group were 0.440±0.045, 0.425±0.020, 0.4217±0.033, 0.322±0.023, respectively (Figure 1). No significant differences were seen among the normal culture group, liposome-alone group, and negative control group in terms of ILK mRNA expression levels ($P>0.05$). Negative siRNA liposomes alone did not affect ILK mRNA expression (Figure 2). Transfected ILK expression

Table 1 *A* value of each group of cells and growth inhibition rate ($\bar{x}\pm s$, %)

Groups	0 hour	12 hours	24 hours	48 hours
Normal cultured	0. 5805±0. 19	0. 5834±0. 24	0. 5850±0. 41	0. 5880±0. 21
Liposome	0. 5762±0. 31 (0. 74)	0. 5618±0. 47(3. 22)	0. 5533±0. 30(4. 69)	0. 5582±0. 34(3. 84)
Negative control	0. 5722±0. 35 (1. 42)	0. 5578±0. 25 (3. 91)	0. 5493±0. 36(5. 37)	0. 5513±0. 22(5. 03)
Transfected	0. 5587±0. 34 (3. 76)	0. 3792±0. 29(34. 68)[a,c]	0. 3743±0. 13(35. 52)[a,c]	0. 3663±0. 14(36. 90)[a,c]

[a]$P<0.05$ experimental group *vs* normal cultured group; [c]$P<0.05$ experimental group at each time point *vs* the 0 hour.

Table 2 Number of cells in the damaged area at different times and the migration ability of cells ($n=12$)

Time	Normal culture group		Transfected group	
	Cells (×400)	Migration capabilities (%)	Cells (×400)	Migration capabilities (%)
0 hour	5. 1667±1. 115	0	4. 9167± 1. 240	0
12 hours	9. 250±1. 485	79. 03[b]	6. 417±1. 165	30. 51[b]
24 hours	27. 167±3. 271	425. 81[b]	10. 333±1. 303	110. 16[b]

[b]$P<0.01$ *vs* normal culture group.

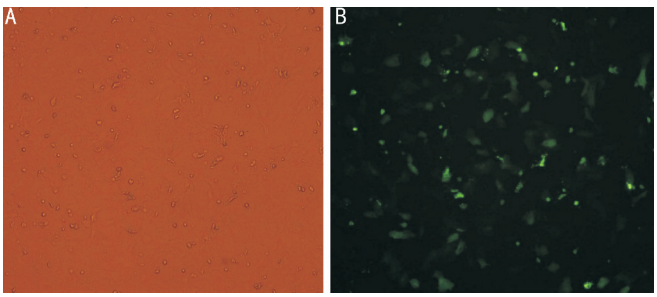

Figure 1 siRNA－ILK (50nmol/L) transfected into HRPE cells(×100). A: Transfected HRPE cells were observed under microscope; B: Transfected HRPE cells were observed under fluorescent microscope at the same eyesight.

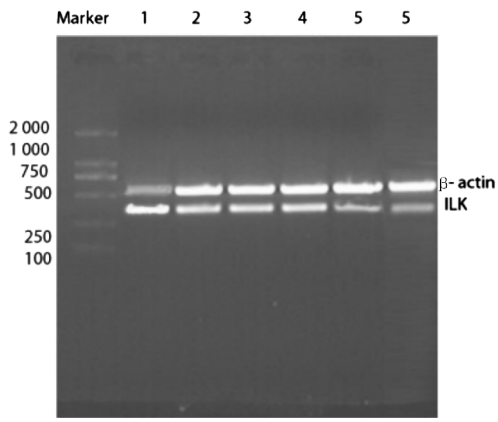

Figure 2 ILK mRNA expression of each groups. Numbers 1, 2, 3, 4 and 5 correspond to the positive control group, the normal culture group, the liposome－alone group, the negative control group, and the transfected group, respectively.

was significantly lower than that in the normal training group ($P<0.01$). In the normal culture group, liposome－alone group, negative control group, and ILK transfection group, the relative gray values were 561. 83 ± 18. 63, 544. 25 ± 23. 27, 547. 75 ± 20. 57 and 325. 13 ± 29. 32, respectively. The number of transfected cells with ILK protein expression was significantly lower than that of normal training cells ($P<0.01$). The three remaining groups did not exhibit statistical significance ($P>0.05$, Figure 3).

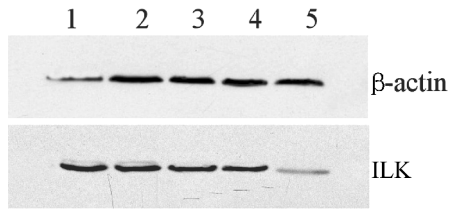

Figure 3 ILK protein expression of each groups. Number 1, 2, 3, 4 and 5 correspond to the positive control group, the normal culture group, the liposome－alone group, the negative control group, and the transfected group group, respectively.

Thiazolyl Blue (MTT) Cell Proliferation Assay Results

We calculated the cell growth inhibition rate (P1) of the transfected group [inhibition rate = (average *A* of normal culture group－average *A* of experimental group)/average *A* of the normal culture group ×100] (Table 1).

Wound healing experiments The single integration of two group RPE cells after scraped part of cells and in 1% FBS cultured, we saw that the cells of enterning into the scratched area of normal cultured group much more increased than in transfected group after 24 hours at low magnification observation. They also formed variable lengths and showed long migration distances. Approximately, 50nmol/L of siRNA－ILK in the transfected RPE cells significantly inhibited migration (Figure 4, Table 2).

Transwell cell migration experiments We scraped a small room of the upper cell membrane of cells before and after Wright－Giemsa dye staining. In the transfected group (Figure 5), the number of perforated cells significantly decreased (16. 17 ± 2. 37) compared with that in the normal culture group (Figure 6, 34. 33 ± 3. 42). The differences were statistically significant ($t=13.43$, $P<0.01$).

DISCUSSION

RPE cells do not proliferate under normal circumstances, Tsuboin *et al*[1] showed that during stimulation of RPE cells after retinal detachment and reentry into the cell cycle, the cells become larger, and migration and proliferation. Thus, the tendency to close the missing area arises. ILK is a cytoplasmic

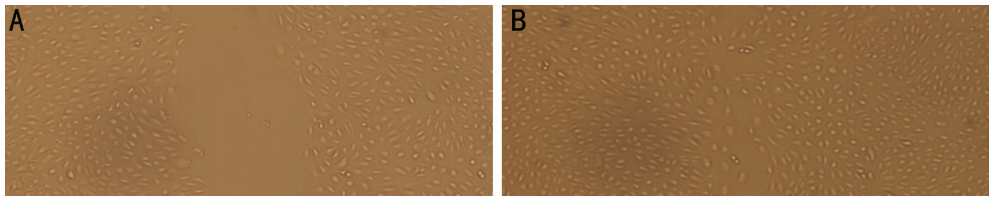

Figure 4 Amount of cells that migrating into the wound area after 24 hours. A: Transfection group; B: The normal culture group, two group cells were scraped at the same wide band at first.

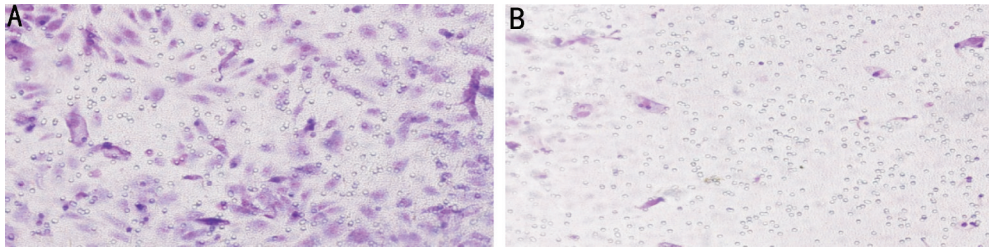

Figure 5 Amount of cells in the transfected group after 24 hours A: Upper cell member; B: After piercing.

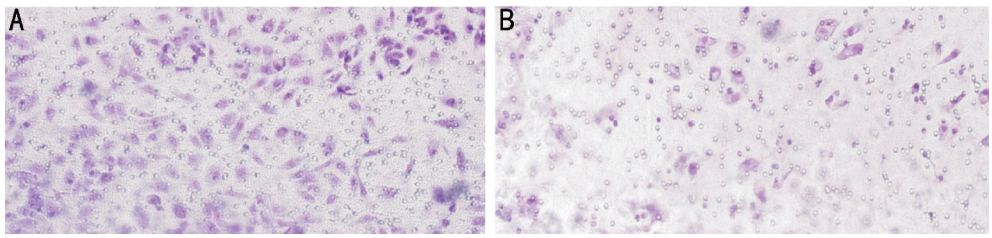

Figure 6 Amount of cells in the control group after 24 hours A: Upper cell member; B: After piercing.

Ser/Thr kinase and is a multifunctional protein involved in multiple signal transduction pathways[2,3], including integrins, growth factors and Wnt signal transduction. It has an important function in the regulation of cell adhesion, apoptosis, spreading, migration, growth, the cell cycle, matrix accumulation, and the formation of new blood vessels[4,5]. ILK mRNA is widely expressed in various normal human tissues. It is a form of low kinase activity that has a physiological role. The serum, growth factor receptor and cell-ECM interactions of co-stimulatory signals influence the kinase activity of ILK in PI3K-dependent manner.

In this experiment, ILK gene transcription and protein expression in the serum-free culture of hRPE cells were detected. We suppose that the retinal tear, retinal trauma and other causes bare, Ilk is activated or increased expression of hRPE cells, RPE cells migrate to the vitreous cavity, and the very active proliferation. ILK in mammalian cells is necessary for Wnt signal transduction. Wnt signaling is to the regulation of cell development and growth with the establishment of cell polarity, cell fate determination and other developmental pathways. It is also important in the other elements of the PI3K signaling pathway. ILK is involved in the receptor signaling pathway by coupling the formation of complexes, such as integrin and growth factor. ILK is associated with the receptors involved in the regulation of cell-based functions, such as extracellular matrix interactions, cell growth and proliferation of intracellular signal transduction. ILK exhibits small changes, besides the changes in its expression and activity, it can affect the signaling pathway and subsequently, the cell cycle, causing abnormal cell growth and proliferation. The ILK pathway has an important function in extracellular matrix degradation. Overexpression of ILK increases the expression of MMP-9, indicating that ILK may play a role in tumor invasion and angiogenesis. Overexpression of ILK in intestinal epithelium and breast epithelium can carry GSK-3β and AP-1 transcription factors to stimulate the expression of matrix metalloproteinase-9. In several brain tumor studies, small molecule inhibitors of ILK can inhibite *in vitro* inhibition of high expression of ILK invasive cells[6].

Studies show that integrins receive signals from the ECM, and are actively involved in ECM assembly, ILK may enhances the Fn-integrin extracellular and intracellular integrin-cytoskeleton interactions that may be involved in ILK from intracellular to extracellular signal transduction, thereby regulating Fn matrix assembly and leading to Fn in extracellular accumulation[7]. Both rat intestinal epithelial cells and mouse mammary epithelium lead to high expressions of ILK in cell-cell adhesion and cellular loss of interaction with reduction in the extracellular matrix[8]. ILK overexpression or persistent activation results in non-adherent cell-dependent survival of tumorigenic transformation, increased tumorigenicity and tumor invasion potential[9].

ILK targeted at segments of RNA interference significantly inhibited of ILK transcription and protein synthesis in hRPE cells. It also significantly inhibited RPE cell proliferation and migration, confirming that *in vitro* transcription for the

synthesis of the ILK targeting specific siRNA can be efficiently reduced in hRPE cells and in the ILK gene, ILK also inhibited hRPE cell proliferation and migration. ILK may participate in various processes after the formation of retinal tears in RPE cell proliferation and migration. ILK as an integrins and growth factor receptor signaling pathway component, is an important factor that regulates cell adhesion, survival, differentiation and apoptosis. Thus, ILK activation is closely related to increased cell proliferation and migration. ILK in retinal pigment cells freed from the hole during abnormal proliferation and its occurrence and development of PVR. ILK is thus expected to become a new target for the treatment of PVR.

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整合素连接激酶对人视网膜色素上皮细胞增殖和迁移的影响

张奕霞[1],张晓阳[2],陈宁宁[1],李彩云[1]
(作者单位:[1]830008 中国新疆维吾尔自治区石河子市,新疆石河子大学医学院第一附属医院眼科;[2]830000 中国新疆维吾尔自治区乌鲁木齐市,新疆医科大学第五附属医院老年病内科)
作者简介:张奕霞,女,副主任医师,研究方向:玻璃体视网膜疾病。
通讯作者:张奕霞. xyz991128@163.com

摘要

目的:探讨 RNA 干扰抑制整合素连接激酶(integrin-linked kinase, ILK)对人视网膜色素上皮(human retinal pigment epithelium,hRPE)细胞增殖和迁移的影响。

方法:体外培养 hRPE 细胞,设计并合成特异性人 ILK 的 RNA 干扰片段,阳离子脂质体转染入人视网膜色素上皮细胞,利用 RT-PCR 及 Western blot 半定量检测 siRNA 对 ILK 基因及蛋白表达的抑制作用,MTT 方法检测转染前后 siRNA 对细胞增殖的抑制作用。损伤愈合实验和 Transwell 实验观察人视网膜色素上皮细胞迁移能力的改变。

结果:培养的 hRPE 细胞存在 ILK 的基因转录表达,siRNA 显著抑制 hRPE 细胞 ILK 的 mRNA 和蛋白表达($P<0.01$)。MTT、损伤愈合实验和 Transwell 实验提示转染 ILK-siRNA 后人视网膜色素上皮细胞的增殖、迁移能力有明显下降,与正常对照组相比有统计学差异($P<0.05$)。

结论:特异性 ILK-siRNA 能有效抑制 ILK 在 hRPE 的 mRNA 和蛋白的表达并显著降低 hRPE 的增殖和迁移能力。

关键词:RNA 干扰;整合素连接激酶;视网膜色素上皮细胞;增殖;迁移