Knockdown of eukaryotic translation initiation factor 4E suppresses cell growth and invasion, and induces apoptosis and cell cycle arrest in a human lung adenocarcinoma cell line

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Received December 18, 2014; Accepted September 23, 2015

DOI: 10.3892/mmr.2015.4468

Abstract. Eukaryotic translation initiation factor 4E (eIF4E) was shown to be upregulated in malignant human tumors. To assess the effect of downregulation of eIF4E on the proliferation and invasiveness of a human lung adenocarcinoma cell line, a short hairpin (sh)RNA targeting eIF4E was constructed and transfected into A549 human lung adenocarcinoma cells. The expression of eIF4E was determined by reverse transcription-quantitative polymerase chain reaction and western blotting. Cell viability was assessed using a Cell Counting kit-8, and apoptosis levels and cell cycle distribution were assessed by flow cytometry. Invasiveness was assessed using Transwell chambers. Transfection of the A549 cells with eIF4E targeting shRNA reduced the mRNA and protein expression levels of eIF4E by >70% 48 and 72 h following transfection, and eIF4E targeting shRNA-transfected cells were significantly less viable compared with the cells transfected with scrambled shRNA. The rate of apoptosis was also significantly increased, significantly more cells were in the G_0/G_1 phase and fewer were in the S phase, indicating cell cycle arrest. The fraction of transfected cells migrating across Transwell inserts were also reduced. In conclusion, inhibition of eIF4E suppressed cell growth and invasion, induced apoptosis and cell cycle arrest, suggesting that eIF4E may be a potential therapeutic target in lung adenocarcinoma.

Introduction

Due to its malignant biological characteristics, including invasion and metastasis, patients with lung cancer usually have a poor prognosis, and in 2012, 1.59 million succumbed to lung cancer worldwide (1). Therapies to combat the growth and metastasis of lung cancers are urgently required. By elucidating the genetic and molecular mechanisms underlying cancer cell proliferation and metastasis, targets for anticancer therapy may be revealed.

Eukaryotic translation initiation factor 4E (eIF4E) has an important role in the regulation of eukaryotic translation initiation (2), and recognizes the 5'-cap of mRNA. The 5'-cap of mRNA has been reported to be associated with mRNA transcription and translation, preventing the degradation of mRNA precursors, and serving as a binding site for regulatory factors, therefore, being involved in various RNA metabolic processes (3). By binding to the 5'-cap of mRNA, eIF4E forms a translation initiation complex involved in protein trafficking and translation, thereby regulating gene expression (4,5).

Previous studies have reported that eIF4E is upregulated in various human malignant tumors, and that eIF4E expression is closely associated with tumorigenesis, infiltration and metastasis in solid tumors, including lung, breast and prostate cancer (6-10). The overexpression of eIF4E is also negatively correlated with survival in advanced cancer types, including head and neck, breast, prostate, lung, and hematologic malignancies (6,8-12). In addition, eIF4E phosphorylation has been reported to be specifically increased in advanced malignancies (7,12,13). Our previous study demonstrated that the expression levels of eIF4E were increased in the tumor, compared with adjacent tissue samples, and its elevated expression was associated with lymph node metastasis (13). Therefore, this led to the hypothesis that eIF4E downregulation may lead to biological function inhibition in lung cancer cells.

The present study sought to further elucidate the mechanisms underlying the effects of eIF4E on cell proliferation, apoptosis, invasion and the cell cycle. The expression of eIF4E was inhibited in A549 lung adenocarcinoma cells using RNA interference, and the impact of eIF4E knockdown on the rate of apoptosis, as well as the capacity of these cells to proliferate, migrate and progress through the cell cycle, was subsequently investigated *in vitro*.

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Key words: lung neoplasms, eukaryotic translation initiation factor 4E, RNA interference, cell cycle, cell invasion

Materials and methods

Materials. DNA restriction enzymes (*Bbs*I, *Pst*I and *Bam*HI) and DNA ligase were purchased from Thermo Fisher Scientific, Inc., (Waltham, MA, USA). A DNA gel purification kit was purchased from Tiangen Biotech Co., Ltd. (Beijing, China), a Plasmid Extraction kit was purchased from Hangzhou Axygen Biotechnology, Ltd. (Hangzhou, China), and the Quantitative fluorescent Polymerase Chain Reaction (PCR) kit was purchased from Shanghai Bio-Tech Co., Ltd (Shanghai, China). Rabbit anti-eIF4E was purchased from Abcam (Cambridge, MA, USA). DNA primers were synthesized by GenePharma Co., Ltd. (Shanghai, China). A Gibco Cell Apoptosis kit was purchased from Thermo Fisher Scientific, Inc., and the Cell Cycle kit from Multi Sciences (Lianke) Biotech Co., Ltd. (Hangzhou, China). The A549 lung adenocarcinoma cell line was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). The pGPU6/green fluorescent protein (GFP)/Neo plasmid was obtained from GenePharma Co., Ltd.

Construction of an eIF4E-short hairpin (sh)RNA plasmid. A total of three sets of primers for eIF4E-directed shRNA and one set of primers for scrambled sequence shRNA were designed by Oligo Designer 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and synthesized by GenePharma Co., Ltd. (Table I). The template contained a TTCAAGAGA loop sequence, a T6 transcription termination sequence, CACC was added to the 5'-end of the sense template to yield a sticky end following *Bbs*I digestion, and GATC was added to the 5'-end of the anti-sense template to yield a complementary sticky end following *Bam*HI digestion. The four oligonucleotides were ligated into a pGPU6/GFP/Neo plasmid following annealing and were termed eIF4E-shRNA1, eIF4E-shRNA2, eIF4E-shRNA3 and NC-shRNA. All plasmid sequences were confirmed by restriction enzyme digestion and sequencing (Thermo Fisher Scientific, Inc.).

Cell culture and transfection. A549 lung adenocarcinoma cells were cultured in RPMI-1640 media (HyClone Laboratories; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories; GE Healthcare Life Sciences), at 37˚C in an atmosphere containing 5% CO_2. The cells were washed with phosphate-buffered saline (PBS) and 0.25% trypsin digestion (Gibco; Thermo Fisher Scientific, Inc.) for passaging. The cells were transfected with Invitrogen Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) alone (negative control), or one of the three eIF4E-targeting shRNA: eIF4E-shRNA1, eIF4E-shRNA2 or eIF4E-shRNA3, according to the manufacturer's protocol. Transfected cells were selected by addition of G418 (Amresco LLC, Solon, OH, USA) to the culture media.

Measurement of the mRNA expression of eIF4E by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was extracted from the A549 cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The cDNA was reverse transcribed, according to the Fermentas M-MLV reverse transcriptase protocol (Thermo Fisher Scientific, Inc.). The primer sequences were as follows: eIF4E, forward: 5'-ACG GAATCTAATCAGGAC-3' and reverse: 5'-CCCACATAG GCAATAC-3'; β-actin, forward: 5'-AAGATGACCCAGATC ATGTTTGA-3' and reverse: 5'-TTAATGTCACGCACGATT TCC-3'. The product length was 287 bp. PCR was conducted using a CFX Connect Real-Time PCR system (Bio-Rad Laboratories, Inc.). The PCR cycling conditions were as follows: 35 cycles of 94˚C for 5 min, 94˚C for 10 sec and 60˚C for 30 sec, and 60˚C for 10 min. The expression levels were measured by SYBR® Green (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Measurement of the protein expression of eIF4E by western blotting. The cells ($1x10^7$ cells/ml) were lysed using lysis buffer (pH 7.4, 50 mM Tris-base, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). The homogenate was subsequently centrifuged at 12,000 x g for 15 min at 4˚C, and the supernatant was retained and preserved at -80˚C until further use. A total of 30 μg protein was separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions and was subsequently transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA) prior to blocking with 5% non-fat milk at room temperature for 2 h. The membranes were subsequently incubated with a rabbit anti-human eIF4E primary antibody (1:1,000; cat. no. AB1126; Abcam) overnight at 4˚C, and were washed three times with Tris-buffered saline containing 0.1% Tween-20 (Amresco LLC). The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:1,000; cat. no. BS13278; Bioworld Technology, Inc., St. Louis Park, MN, USA) for 2 h at room temperature. The protein bands were detected using an Enhanced Chemiluminescence kit (Beyotime Institute of Biotechnology) and X-ray exposure (Beyotime Institute of Biotechnology). Densitometry was quantified by Quantity One software (ChemiDoc XRS+ system; Bio-Rad Laboratories, Inc.) and the eIF4E/GAPDH expression level ratio was calculated.

Measurement of cell viability using a Cell Counting kit-8 assay. A549 cells in the logarithmic phase were resuspended and seeded into a 96-well plate at $2x10^3$ cells/well and incubated at 37˚C in an atmosphere containing 5% CO_2. After 24 h, the cells were transfected, as previously described (14). The transfected cells were cultured and after 48 and 72 h, cell viability was assessed using a Cell Counting kit-8 (Beyotime Institute of Biotechnology), and calculated using the following formula: Viability (%) = ($A_{experimental}$ / $A_{control}$) x100%.

Measurement of apoptosis using annexin V staining. The transfected A549 cells in the logarithmic phase were resuspended and seeded into a 6-well plate at $2x10^5$ cells/well. After 24 h, serum-free RPMI-1640 media was added and following a further 24, 48 or 72 h incubation, the cells were resuspended with trypsin and centrifuged at 400 x g for 5 min at room temperature. The cell pellet was washed twice with cold PBS and resuspended in 100 μl 1X Binding Buffer (Taizhou Hospital Laboratory, Linhai, China) to which 5 μl fluorescein

Table I. shRNA sequences used in the present study.

shRNA	Target location	Target sequence	shRNA synthetic sequences
eIF4E-shRNA1	2094	CCAAAGATAGTGATTGGTTAT	Sense: 5'-CACCGCCAAAGATAGTGATTGGTTATTTCAAGAGAATAACCAATCACTATCTTTGGTTTTTTG-3' Antisense: 5'-GATCCAAAAAACCAAAGATAGTGATTGGTTATTCTCTTGAAATAACCAATCACTATCTTTGGC-3'
eIF4E-shRNA2	1849	GGAGGACGATGGCTAATTACA	Sense: 5'-CACCGGAGGACGATGGCTAATTACATTCAAGAGATGTAATTAGCCATCGTCCTCCTTTTTTG-3' Antisense: 5'-GATCCAAAAAAGGAGGACGATGGCTAATTACATCTCTTGAATGTAATTAGCCATCGTCCTCC-3'
eIF4E-shRNA3	1970	GTGGCGCTGTTGTTAATGTTA	Sense: 5'-CACCGTGGCGCTGTTGTTAATGTTATTCAAGAGATAACATTAACAACAGCGCCACTTTTTTG-3' Antisense: 5'-GATCCAAAAAAGTGGCGCTGTTGTTAATGTTATCTCTTGAATAACATTAACAACAGCGCCAC-3'
NC-shRNA			Sense: 5'-CACCGTTCTCCGAACGTGTCACGTCAAGAGATTACGTGACACGTTCGGAGAATTTTTTG-3' Antisense: 5'-GATCCAAAAAATTCTCCGAACGTGTCACGTAATCTCTTGACGTGACACGTTCGGAGAAC-3'

shRNA, short hairpin RNA; eIF4E, eukaryotic initiation of transcription factor 4E; NC, negative control.

Table II. mRNA and protein expression levels of eIF4E in transfected cells.

Treatment	Relative expression levels	
	mRNA	Protein
Mock	0.918±0.150	0.857±0.069
NC	0.905±0.068	0.829±0.102
eIF4E-shRNA1	0.541±0.092[ab]	0.495±0.093[ab]
eIF4E-shRNA2	0.428±0.137[ab]	0.339±0.080[ab]
eIF4E-shRNA3	0.361±0.083[ab]	0.254±0.086[ab]

mRNA expression was assessed by reverse transcription-quantitative polymerase chain reaction and normalized against β-actin. The protein expression was assessed by western blotting and normalized against GAPDH. [a]P<0.01, vs. the mock shRNA; [b]P<0.01, vs. the NC shRNA. shRNA, short hairpin RNA; eIF4E, eukaryotic initiation of transcription factor 4E; NC, negative control.

isothiocyanate-conjugated annexin V antibody and 1 μl propidium iodide (Gibco; Thermo Fisher Scientific, Inc.) was added. The mixture was incubated in the dark for 15 min at 4˚C and cell apoptosis was examined by flow cytometry (Accuri C6; BD Biosciences, San Jose, CA, USA).

Assessment of cell cycle distribution by flow cytometry. Transfected A549 cells in the logarithmic phase were resuspended and seeded into a 6-well plate at $2x10^5$ cells/well. After 24 h, serum-free RPMI-1640 media was added. Following a further 24, 48 or 72 h incubation, the cells were resuspended in trypsin and centrifuged at 400 x g for 5 min at room temperature. The cell pellet was resuspended and fixed in 75% ethanol at 4˚C for 24 h. The sample was subsequently centrifuged at 400 x g for 5 min at room temperature and the cell pellet was air dried. The dry pellet was washed twice with PBS and resuspended in 1 ml reagent A [Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China], and vortexed for 5-10 sec. After 30 min, the cell cycle was examined by flow cytometry (Accuri C6; BD Biosciences).

Assessment of cell invasion using a Transwell assay. Matrigel (BD Biosciences) dissolved at 4˚C was diluted in cold PBS. A total of 100 μl diluted gel (~25 μg) was added to the upper chamber of each well of a 24-well Transwell plate. The plate was incubated overnight at 37˚C for basal lamina coating and washed with serum-free RPMI-1640 media. The transfected cells were resuspended in RPMI-1640, supplemented with 1% FBS at $5x10^4$/ml, and a 200 μl suspension was added into the upper chamber and 600 μl culture medium was added to the lower chamber. The plate was incubated for 24 h at 37˚C in an atmosphere containing 5% CO_2. Non-migrating cells in the upper chamber were removed with a cotton swab. The Transwells were removed and the plate was inverted and air dried. A total of 500 μl 0.1% crystal violet solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well for 30 min at 37˚C and the Transwell plate was subsequently washed with PBS. Four fields were selected for cell counting.

Statistical analysis. The data were presented as the mean ± standard deviation. Frequencies were compared by Student's t-test, and comparisons between groups were made by one-way analysis of variance. The data were analyzed using the SPSS 17.0 software package (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of shRNA transfection on the mRNA and protein expression levels of eIF4E. A549 lung adenocarcinoma cells were transfected with one of three eIF4E-targeting shRNAs or scrambled shRNA. RT-qPCR revealed that the mRNA expression of eIF4E in the cells transfected with scramble shRNA (0.905±0.068 relative to β-actin mRNA) revealed no significant difference compared with those in the mock transfected control cells (0.918±0.150). However, the relative mRNA expression levels of eIF4E in cells transfected with eIF4E-shRNA1, eIF4E-shRNA2 and eIF4E-shRNA3 were significantly reduced to 0.5±0.2, 0.4±0.2, 0.3±0.1, respectively (P<0.01; Table II).

Western blotting revealed that the reduction in mRNA eIF4E expression levels corresponded to a significant reduction in the protein expression levels of eIF4E in cells transfected with all three eIF4E-targeting shRNAs (P<0.01; Table II). eIF4E-shRNA3 reduced the eIF4E protein expression levels most significantly relative to those of GAPDH, from 0.857±0.069 in the mock transfected cells, to 0.254±0.086 (P<0.01; Table II). Therefore, eIF4E-shRNA3 was used for further experimentation.

Effects of eIF4E-shRNA on cell viability. Although mock or scrambled shRNA transfection caused no impact on cell viability, the viability of eIF4E-shRNA-transfected cells was significantly lower compared with that of mock transfected cells after 48 and 72 h (48 h post-transfection, 94.9±3.3%, 89.0±3.8%, P<0.05; 72 h post-transfection, 96.1±2.8%, 88.6±2.4%, P<0.05; Fig. 1).

Transfection of scrambled shRNA significantly increased the rate of apoptosis from 1.43±0.29% in the untreated cells and 1.53±0.25% in the mock transfected cells to 1.60±0.10% 48 h post-transfection with scrambled shRNA. However, transfection with eIF4E-shRNA induced a significantly higher rate of apoptosis (3.57±0.42% after 48 h and 4.63±1.30 after 72 h; P<0.05; Fig. 2).

Effects of eIF4E-shRNA on the cell cycle. Although mock and scrambled shRNA transfection caused no impact on the cell

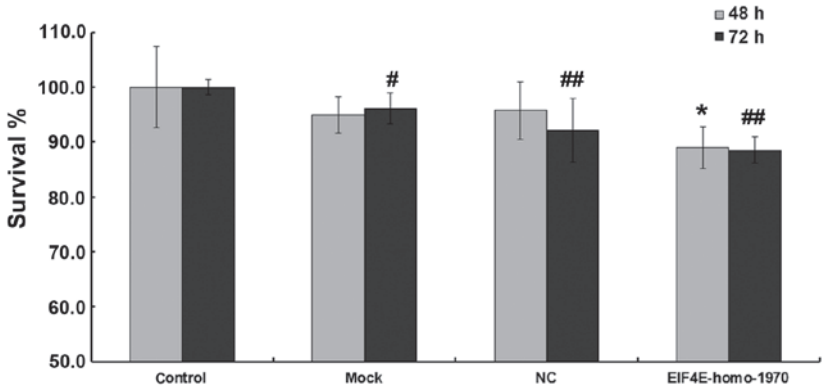

Figure 1. Transfection of scrambled shRNA inhibits the cell viability. Downregulation of eIF4E by targeted shRNA significantly suppressed the cell viability after 48 and 72 h (*P<0.05 and #P<0.05, vs. the mock group). shRNA, short hairpin RNA; eIF4E, eukaryotic initiation of transcription factor 4E; NC, negative control.

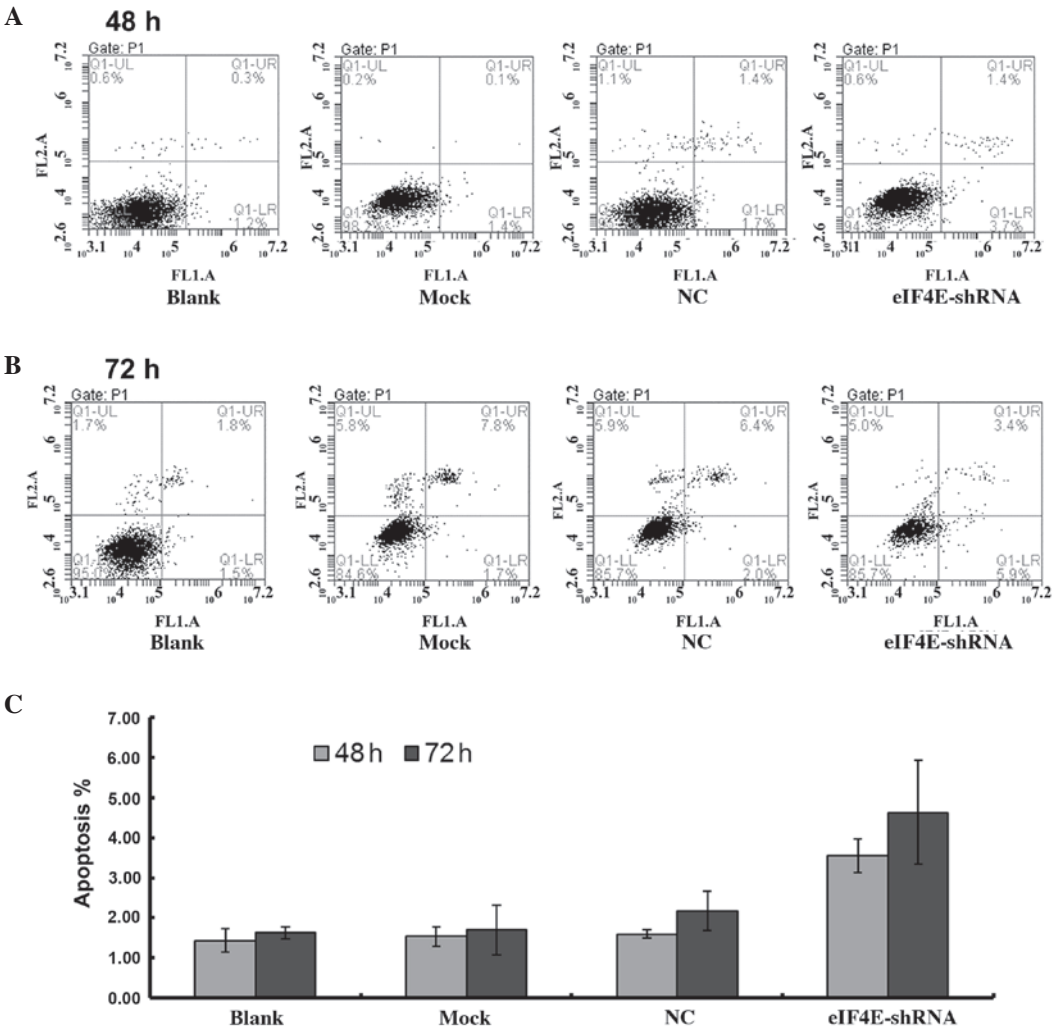

Figure 2. Transfection of scrambled shRNA induces cell apoptosis. Downregulation of eIF4E by targeted shRNA after (A) 48 h and (B) 72 h significantly increased cell apoptosis. (C) Quantification of cell apoptosis rates (*P<0.05 and #P<0.05 vs. the mock group). shRNA, short hairpin RNA; eIF4E, eukaryotic initiation of transcription factor 4E; NC, negative control.

cycle, the fraction of eIF4E-shRNA transfected cells in the G_0/G_1 phase was significantly increased, whereas the percentage of cells in the S phase was reduced, indicating G_0/G_1 arrest. The fraction of cells in the G_0/G_1 phase increased from 49.08±2.57 in the scrambled shRNA-transfected cells to 57.14±0.59 after 48 h. This changed from 64.87±2.45 before to 73.95±6.00 after 72 h (P<0.05; Table III). The fraction of cells in the S phase decreased from 45.81±1.32 in the scrambled shRNA-transfected cells to 23.81±0.83 after 48 h, and from 25.75±2.07 to 14.29±1.75 after 72 h (P<0.05, Table III).

Table III. Cell cycle distribution following transfection.

Treatment	48 h post-transfection			72 h post-transfection		
	G_0/G_1 (%)	S (%)	G_2/M (%)	G_0/G_1 (%)	S (%)	G_2/M (%)
Untreated	46.88±1.67	38.06±2.10	15.06±1.18	63.10±2.03	28.29±2.83	8.61±1.21
Mock	48.84±1.64	41.64±1.57	9.52±1.92	63.33±1.28	27.98±1.09	8.69±2.34
NC	49.08±2.57	45.81±1.32	5.11±0.96	64.87±2.45	25.75±2.07	9.38±1.70
eIF4E-shRNA	57.14±0.59[a]	23.81±0.83[a]	19.05±1.78	73.95±6.00[a]	14.29±1.75[a]	11.76±2.74

Cell cycle was detected by flow cytometry. [a]P<0.05, vs. the untreated, mock and NC group. shRNA, short hairpin RNA; eIF4E, eukaryotic initiation of transcription factor 4E; NC, negative control.

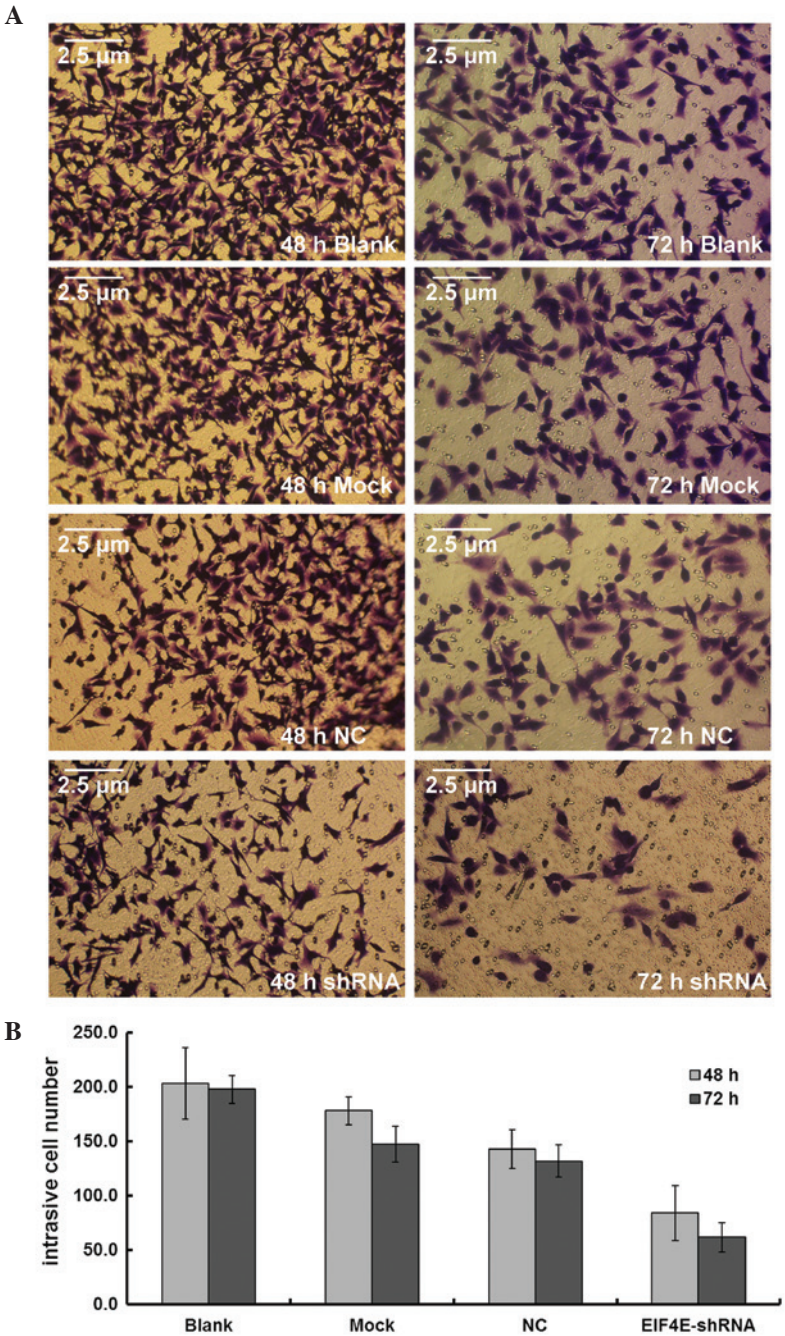

Figure 3. Transfection of scrambled shRNA suppresses cell invasion (magnification, x400). (A and B) Cell invasive potential, indicated by Transwell assay, was markedly weakened in the eIF4E-shRNA group after 48 and 72 h (*P<0.05 and $^\#$P<0.05, vs. the mock group). shRNA, short hairpin RNA; eIF4E, eukaryotic initiation of transcription factor 4E; NC, negative control.

Effects of eIF4E-shRNA on cell invasion. Although mock and scrambled shRNA transfection revealed no impact on the number of cells transversing a Transwell insert, the fraction of eIF4E-shRNA-transfected cells migrating was significantly lower. The fraction of cells migrating decreased from 182.5±14.0 in the scrambled shRNA-transfected cells to 83.8±25.4 in the eIF4E-shRNA-transfected cells after 48 h, and from 167.5±30.4 to 61.5±13.5 after 72 h ($P<0.01$; Fig. 3).

Discussion

Due to propensity of lung cancer to invade and metastasize, patients with lung cancer usually have a poor prognosis (1,15). Lung cancer is a major cause of mortality worldwide (1), and therefore, investigations into the genetic contributors to the cell proliferation and metastasis of lung cancer are urgently required.

Previous studies reported that the expression of eIF4E was upregulated in malignant human tumors (2,6). The present study aimed to further elucidate the mechanisms underlying the effects of eIF4E on cell proliferation, apoptosis, invasion and cell cycle. Several constructs were successfully cloned to harbor eIF4E shRNAs targeting various eIF4E domains. Transfection of A549 cells with eIF4E-targeting shRNA reduced the mRNA and protein expression levels of eIF4E by >70%.

A549 cells transfected with eIF4E-targeting shRNA were significantly less viable compared with A549 cells transfected with scrambled shRNA. The rate of apoptosis was also significantly increased, and significantly more cells were in the G_0/G_1 phase and fewer were in the S phase, indicating cell cycle arrest. These observations supported previous studies that demonstrated that eIF4E selectively enhanced the translation and nucleocytoplasmic transport of mRNAs containing long, highly structured untranslated regions, generally encoding proteins involved in the cell growth (cyclin D1, c-myc), angiogenesis [vascular endothelial growth factor (VEGF), fibroblast growth factor-2], invasion [matrix metalloproteinase (MMP)-9, heparanase], and survival (survivin, B cell lymphoma 2) (6,11,16-19). The fraction of transfected cells migrating across the Transwell inserts was also reduced, likely due to the regulation of cell migration-associated genes, including Cyclin D1, VEGF, and MMP-9 by eIF4E (6). These results suggested that the overexpression of eIF4E contributes to the proliferative phenotype of the A549 lung adenocarcinoma cell line, and that eIF4E is crucial for cell cycle progression and invasion.

In vitro, eIF4E phosphorylation appears to be important for the proliferation of numerous tumor cell lines (20-22), and the upregulation of eIF4E expression in transgenic mice increased the incidence of lymphoma, lung adenocarcinoma, angiosarcoma and hepatoma (23,24). Conversely, disruption of eIF4E phosphorylation inhibits tumor development in mouse lymphoma and prostate cancer models (25,26).

As the activity levels of eIF4E are elevated in cancer cells, cancer may be preferentially susceptible to eIF4E-targeted therapy. Several eIF4E inhibitors have recently been investigated for clinical use, including an eIF4E-targeting antisense oligonucleotide (27,28), and small molecular inhibitors of eIF4E phosphorylation or activity (29,30), including mRNA cap structure binding (31,32) and eIF4E:eIF4G binding (33).

In conclusion, the results of the present study indicated that the inhibition of eIF4E may suppress A549 cell growth and invasion, and induce apoptosis and cell cycle arrest *in vitro*, demonstrating a critical role for eIF4E in lung cancer cell growth. As numerous studies (34-36) have now implicated eIF-4E overexpressison in a wide range of human tumor types, and eIF-4E overexpression has been associated with disease progression, strategies to decrease eIF4E expression may represent a potential therapeutic technique in numerous cancer types. These findings indicated that strategies to decrease eIF4E expression or inhibit eIF4E function represent a promising strategy for reducing the rate of lung adenocarcinoma proliferation and metastasis.

Acknowledgements

The present study was supported by the Zhejiang Medical and Health Science and Technology Plan Project (no. 2014KYB310) and the Taizhou Science and Technology Plan Project (no. 11ky09).

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