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Down-regulation of eIF5A-2 prevents epithelial-mesenchymal transition in non-small-cell lung cancer cells*

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Abstract: Background: Epithelial-mesenchymal transition (EMT) is believed to be the critical process in malignant tumor invasion and metastases, and has a great influence on improving the survival rate in non-small-cell lung cancer (NSCLC) patients. Recent studies suggested that eukaryotic initiation factor 5A-2 (eIF5A-2) might serve as an adverse prognostic marker of survival. We detected eIF5A-2 in NSCLC A549 cells, and found that the invasive capability correlates with the eIF5A-2 expression. Methods: Transforming growth factor (TGF)-β1 was used to induce EMT in A549 cells. Western blotting, immunofluorescence, wound healing assay, and transwell-matrigel invasion chambers were used to identify phenotype changes. Western blotting was also used to observe changes of the expression of eIF5A-2. We down-regulated the eIF5A-2 expression using an eIF5A-2 siRNA and identified the phenotype changes by western blotting and immunofluorescence. We tested the change of migration and invasion capabilities of A549 cells by the wound healing assay and transwell-matrigel invasion chambers. Results: After stimulating with TGF-β1, almost all A549 cells changed to the mesenchymal phenotype and acquired more migration and invasion capabilities. These cells also had higher eIF5A-2 protein expression. Down-regulation of eIF5A-2 expression with eIF5A-2 siRNA transfection could change the cells from mesenchymal to epithelial phenotype and decrease tumor cell migration and invasive capabilities significantly. Conclusions: The expression of eIF5A-2 was up-regulated following EMT phenotype changes in A549 cells, which correlated with enhanced tumor invasion and metastatic capabilities. Furthermore, in the A549 cell line, the process of EMT phenotype change could be reversed by eIF5A-2 siRNA, with a consequent weakening of both invasive and metastatic capabilities.

Key words: Non-small-cell lung cancer (NSCLC), Epithelial-mesenchymal transition (EMT), Eukaryotic initiation factor 5A-2 (eIF5A-2), Transforming growth factor (TGF)-β1, A549

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1 Introduction

Lung cancer is one of the most aggressive malignancies, and is the primary cause of cancer death worldwide (Jemal *et al.*, 2008). Approximately 80% of lung cancers are pathologically classified as non-small-cell lung cancers (NSCLCs), which is associated with low five-year survival rates, less than 5% in the presence of metastatic disease (Jemal *et al.*, 2008). The main cause of NSCLC is not fully understood. Surgery is the preferred treatment for the early stage of NSCLC (Wozniak and Gadgeel, 2007), but 30%–40% of patients present at an advanced stage,

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and chemotherapy is the main treatment option for this group. Despite many clinical studies, five-year survival rates for patients with lung cancer remain low. Recurrent and metastatic disease is the main cause of death (Rosell *et al.*, 2004). Hence, an understanding of the molecular mechanism of tumor recurrence and metastases, and the identification of relevant markers and targets are important for improving outcomes in NSCLC.

At present, epithelial-mesenchymal transition (EMT) is the critical process in malignant tumor invasion and the development of metastases (Thiery, 2002). During EMT, epithelial cells lose their epithelial properties, including cell polarity and connection with the basal membrane, and obtain mesenchymal properties, such as higher migration and invasion capabilities, anti-apoptosis activity, and the ability to degrade extracellular matrix. In addition, Thiery (2002) demonstrated that loss of the epithelial marker E-cadherin may be associated with disease progression, the development of metastases, and a poor prognosis in patients with lung cancer.

Recent studies have confirmed the expression of the eukaryotic initiation factor 5A-2 (eIF5A-2) gene in NSCLC. He *et al.* (2011) first reported a correlation between overexpression of eIF5A-2 and local invasion in NSCLC, and suggested that eIF5A-2 might serve as an adverse prognostic marker of survival in stage I disease. As an upstream regulatory factor in protein synthesis, eIF5A has been well established as an essential factor for sustained cell proliferation in mammalian cells (Clement *et al.*, 2003; 2006; Li *et al.*, 2004). Accelerated growth and division of cells depend on the up-regulation of the protein synthetase system. Almost all relevant invasive gene mRNA expression is controlled post-transcriptionally. Any minor change in the translation factor at this level will cause an imbalance, with consequent variations in cell behavior (Tang *et al.*, 2010). Inhibition of eIF5A activation exerts strong anti-proliferative effects in various human cancer cell lines and causes arrest of cell cycle progression (Clement *et al.*, 2002; Nishimura *et al.*, 2005).

As a member of the eIF5A family, overexpression of eIF5A-2 might result in changes in some EMT relevant factors, such as a decrease in E-cadherin and an increase in vimentin, which might lead to enhanced invasive and metastatic capabilities. The current study focuses on the role of eIF5A-2 in NSCLC, particularly the mechanisms by which eIF5A-2 regulates EMT, which causes tumor invasion and metastatic disease.

2 Materials and methods

2.1 Cell and cell culture

The human NSCLC cell line A549 was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and saved according to the ATCC instructions. All cells were cultured in RPMI-1640 medium (Gibco, USA), supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (Sigma, USA), and 10% fetal bovine serum (FBS; Gibco, USA). The cells were maintained at 37 °C in 5% CO_2 and 95% air. To induce A549-EMT cells, transforming growth factor (TGF)-β1 (10 ng/ml) (PeproTech, USA) was added to the medium and the A549 cells were cultured for 48 h.

2.2 Transfections

The A549 cells were transfected with eIF5A-2 siRNA (10 μmol/ml) (Santa Cruz Biotechnology, USA) using Lipofectamine-2000 (Invitrogen, USA), according to the manufacturer's instructions. Negative siRNA (Invitrogen, USA) was used as a non-specific control siRNA, and the transfection procedure was similar to that used for eIF5A-2 siRNA, as described above. The medium was changed with culture medium 6 h after transfection. All experiments were repeated three times.

2.3 Western blotting

The cells were washed twice in ice-cold phosphate buffer solution (PBS) and resuspended in 100 μl cell lysis buffer (Cell Signaling, USA) with protease inhibitors (Sigma, USA). The protein concentration was quantified using BCA protein assay kit (Thermo, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10%) was used to separate cell lysates (40 μg/lane), and then proteins were transferred to polyvinyllidenediflouride (PVDF) membranes (Millipore, USA), blocked with Tris-buffered saline (TBS) and 0.1% Tween 20 (TBS/T) containing 5% bovine serum albumin (BSA), and then incubated with anti-E-cadherin,

anti-vimentin (Biovision, USA) or anti-eIF5A-2 (PeproTech Group, USA) antibodies (1:1000) at 4 °C overnight. The membranes were washed three times with TBS/T and incubated with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The protein bands were developed using chemiluminescence (GE Healthcare, USA) and visualized using autoradiography on X-ray film (Kodak, USA). Band densities were estimated using Image-Pro Plus 6.0 software (Media Cybernetics Inc., USA) and the relative protein expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.4 Wound healing assay

The A549 cells were seeded into six-well plates at a density of 2×10^5 cells/well and cultured with RPMI-1640 medium (containing 10% FBS) overnight. The medium was then changed to RPMI-1640 without FBS and cultured for 24 h until the monolayer adherent cell confluence was >90%. A plastic 100-μl tip was used to scrape a wound across the cell monolayer. The cells were then treated with TGF-β1 (10 ng/ml) for 24 h after transfection with eIF5A-2 siRNA (10 μmol/ml) or negative siRNA (10 μmol/ml) for 6 h. Photomicrographs were taken with an inverted phase contrast microscope (Olympus, Japan), at zero time point and after 24 h, to measure the ratio of the remaining wound area relative to the initial wound area. Image-Pro Plus 6.0 software was used to quantify the wound area.

2.5 Transwell-matrigel invasion assay

Cells were treated with TGF-β1 (10 ng/ml) for 48 h, the treated cells with eIF5A-2 siRNA (10 μmol/ml) or negative siRNA (10 μmol/ml) for another 6 h. The cells were seeded at a density of 5×10^4 cells/well in the upper chamber of a 24-insert with serum-free RPMI-1640 medium. The upper chambers were coated with matrigel (BD Biosciences, USA), and the lower chamber contained 10% FBS plus RPMI-1640 medium. After 24 h, the bottom of the 24-insert was fixed using methanol for 10 min and then stained with hematoxylin and eosin (H&E). The invading cells were measured using an inverted phase contrast microscope and photographed.

2.6 Immunofluorescence

Cells were treated with TGF-β1 (10 ng/ml) for 48 h, eIF5A-2 siRNA (10 μmol/ml), or negative siRNA (10 μmol/ml) for 6 h. The cells were washed with ice-cold PBS and fixed in 4% paraformaldehyde for 30 min followed by incubation with 3% H_2O_2 for 15 min at 37 °C. Serum was then used to block for a further 15 min. Following overnight incubation at 4 °C with anti-E-cadherin, anti-vimentin, or anti-eIF5A-2 antibodies (1:1000), the cells were washed with ice-cold PBS followed by incubation for 1 h at room temperature with the appropriate secondary antibodies conjugated to goat anti-mouse horseradish peroxidase (1:2000). 4,6-diamidino-2-phenylindole (DAPI) (Sigma, USA) was used to mark the nucleus, which was then observed using a fluorescence confocal microscopy (Olympus, Japan).

2.7 Statistical analyses

Data were analyzed with SPSS software (Version 15.0 for Windows, USA) and expressed as mean±standard error of the mean (SEM), using Student's *t*-tests for two-group comparisons. $P<0.05$ is considered statistically significant.

3 Results

3.1 A549 cells underwent EMT phenotype changes on exposure to TGF-β1

It has been reported that chronic exposure to TGF-β1 can induce EMT in A549 NSCLC cells (Maitah *et al.*, 2011). In this study, after exposure to TGF-β1 (10 ng/ml) for 48 h, we found obvious changes in the cell morphology of the A549 cells. All cells changed to the mesenchymal phenotype, as evidenced by an elongated appearance, irregular shape, pseudopodia, and loose of cell-cell junctions.

3.2 TGF-β1 influenced the expression of E-cadherin and vimentin in A549 cells

Apart from changes in the cell morphology, changes in the expression of some EMT markers, such as E-cadherin and vimentin, are also used to confirm the mesenchymal phenotype. Following stimulation by TGF-β1 (10 ng/ml) for 48 h, we tested two typical molecular markers of A549 cells through western blotting. The results show that the epithelial

E-cadherin was markedly down-regulated, and the mesenchymal marker vimentin was markedly up-regulated compared to the A549 parental cells (Fig. 1a). Furthermore, the results of immunofluorescence reveal similar results, with A549-EMT cells demonstrating an almost complete absence of the green E-cadherin expression, while the red vimentin expression was much greater than that observed in the A549 parental cells (Fig. 1b).

3.3 Migration and invasion capabilities of A549 cells were enhanced after stimulation by TGF-β1

Studies have shown that tumor cells demonstrate enhanced migration and invasion capabilities following EMT. In this study, we compared the migration capability of A549 cells before and after exposure to TGF-β1, using a wound healing assay. After exposure to TGF-β1 (10 ng/ml) for 24 h, there was almost complete wound healing, evidenced by a rate of (73.7±1.2)%, while in cells with no TGF-β1 exposure, only a few cells crossed over the wound edge, and the rate of wound healing was (45.0±3.7)% (P<0.05), suggesting that exposure to TGF-β1 can enhance the migration capability of A549 cells (Fig. 2a). In addition, we also used transwell-matrigel invasion chambers to test the invasive capability of A549-EMT cells. More cells invaded through the matrigel after induction by TGF-β1, compared with cells without TGF-β1 stimulation (P<0.05) (Fig. 2b). The results show that the A549-EMT cells have a greater capacity to invade after exposure to TGF-β1 than the A549 parental cells.

3.4 A549-EMT cells had higher expression of the eIF5A-2 protein

To further investigate how TGF-β1 induces A549 cell EMT, we focused on the effects of the eIF5A-2 oncogene in this transition, as eIF5A-2 overexpression might have some connection with EMT that then leads to tumor invasion and the development of metastases. Compared to normal A549 cells, eIF5A-2 protein expression was markedly up-regulated in the A549-EMT cells (Fig. 3). When eIF5A-2 expression was knocked down by eIF5A-2 siRNA, A549 cells were prevented from transitioning into A549-EMT cells. Following stimulation of A549 cells with TGF-β1 (10 ng/ml) for 48 h, after eIF5A-2 siRNA6 (10 μmol/ml) transfection for 6 h, all cells maintained the epithelial morphology. However, when negative siRNA was transfected into A549 cells, all cells transitioned to a mesenchymal morphology. Furthermore, western blotting revealed similar results. When A549 cells were transfected with eIF5A-2 siRNA, E-cadherin expression was increased and vimentin expression decreased significantly, compared to cells transfected with negative siRNA (Fig. 4a). These results demonstrate that overexpression of eIF5A-2 might be one important factor in terms of promoting A549 NSCLC cells to undergo EMT. In addition, the immunofluorescence results revealed more obvious green E-cadherin expression and reduced red vimentin expression, compared to the negative siRNA group (Fig. 4b).

3.5 Down-regulation of eIF5A-2 expression in A549 cells could decrease tumor cell migration and invasive capabilities

We have shown that interfering eIF5A-2 expression in A549 cells could change EMT phenotype clearly. Using the wound healing assay, we found that, when transfected with eIF5A-2 siRNA, the wound healing rate in the A549 cells was only (46.3±3.7)%, which was clearly lower than that observed in the negative siRNA group (Fig. 5a). The transwell-matrigel invasion assay showed that the number of cells in the high power lens of each field of vision was only 602±78, which was significantly less than that observed in the negative siRNA group (P<0.05) (Fig. 5b). These results suggest that following a knockdown of eIF5A-2 expression in A549 cells, cell behavior can change quite markedly, and can decrease the metastatic and invasive capabilities of tumor cells.

4 Discussion

The development of tumor recurrence and metastases is a complicated process, which involves the activation of many oncogenes and the inactivation of many anti-oncogenes. Some oncogene activation might promote disease recurrence and metastases in patients with NSCLC. eIF5A-2 is a newly discovered oncogene of the eIF5A family and demonstrates weak expression in the testis and brain cells, but its overexpression has been associated with the presence of

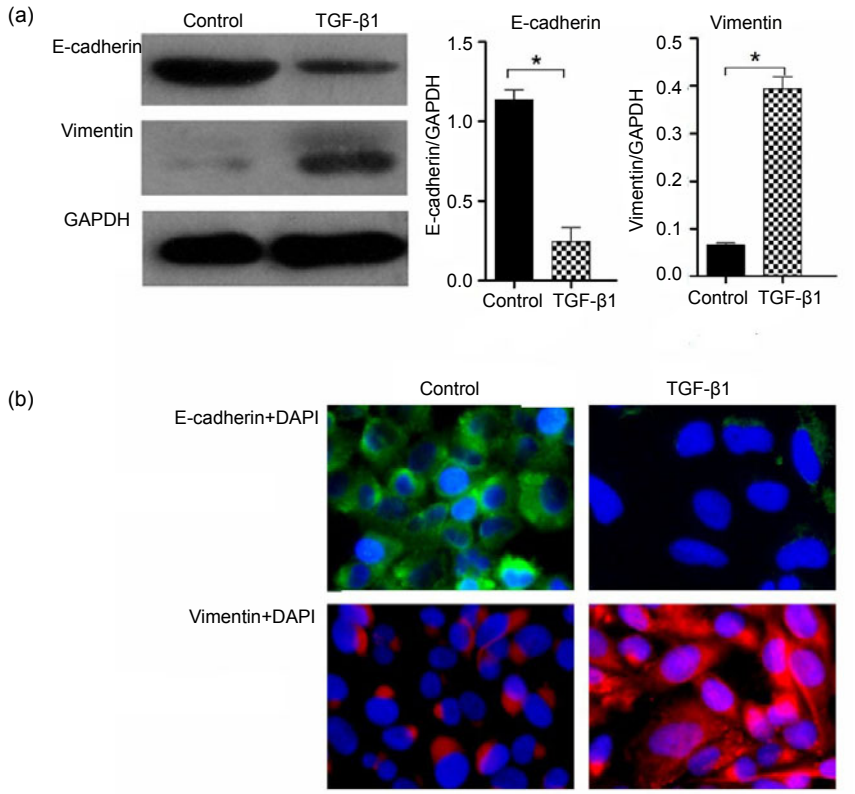

Fig. 1 EMT phenotype changes in A549 cells on exposure to TGF-β1 (10 ng/ml) for 48 h

(a) Western blotting of A549 cells before and after treatment with TGF-β1 for the expressions of E-cadherin and vimentin; (b) Immunofluorescence showed the expressions of E-cadherin and vimentin in A549 cells after inducing by TGF-β1. Data in histogram represent mean±SEM from three independent experiments; * $P<0.05$ by t-test

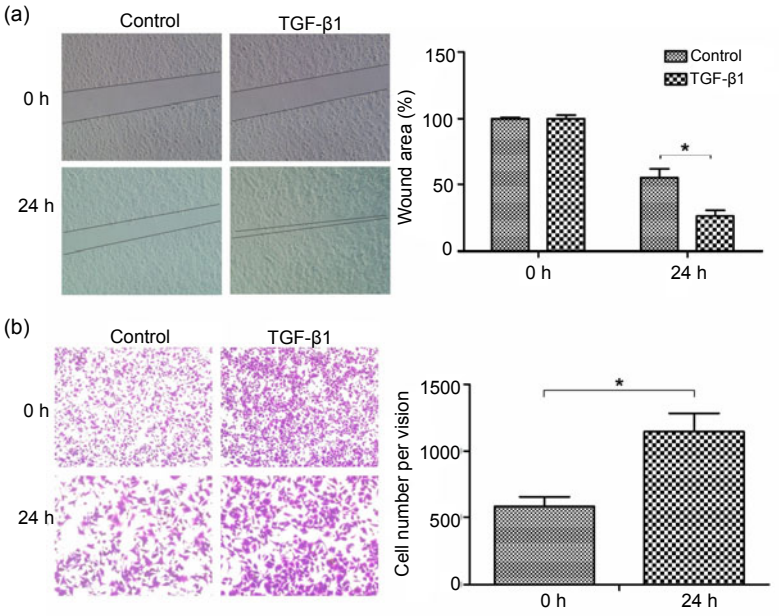

Fig. 2 Enhanced migration and invasion capabilities of A549 cells after stimulation by TGF-β1 (10 ng/ml) for 24 h

(a) Wound healing assay: after exposure to TGF-β1, more A549 cells migrated into the wound area and there was almost complete wound healing, while in the control cells, significantly less cells crossed over the wound edge; (b) Transwell-matrigel invasion assay: more cells invaded through the matrigel after inducing by TGF-β1 compared with the control cells. Data in histogram represent mean±SEM from three independent experiments; * $P<0.05$ by t-test

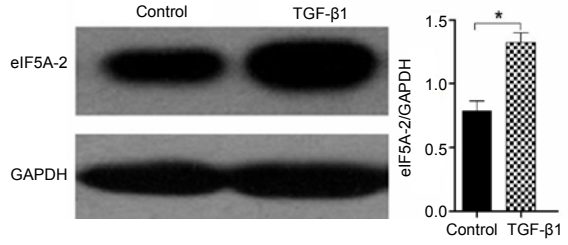

Fig. 3 Western blotting results of the expression of eIF5A-2 protein in A549 cells before (control) and after stimulation by TGF-β1

Data in histogram represent mean±SEM from three independent experiments; * $P<0.05$ by t-test

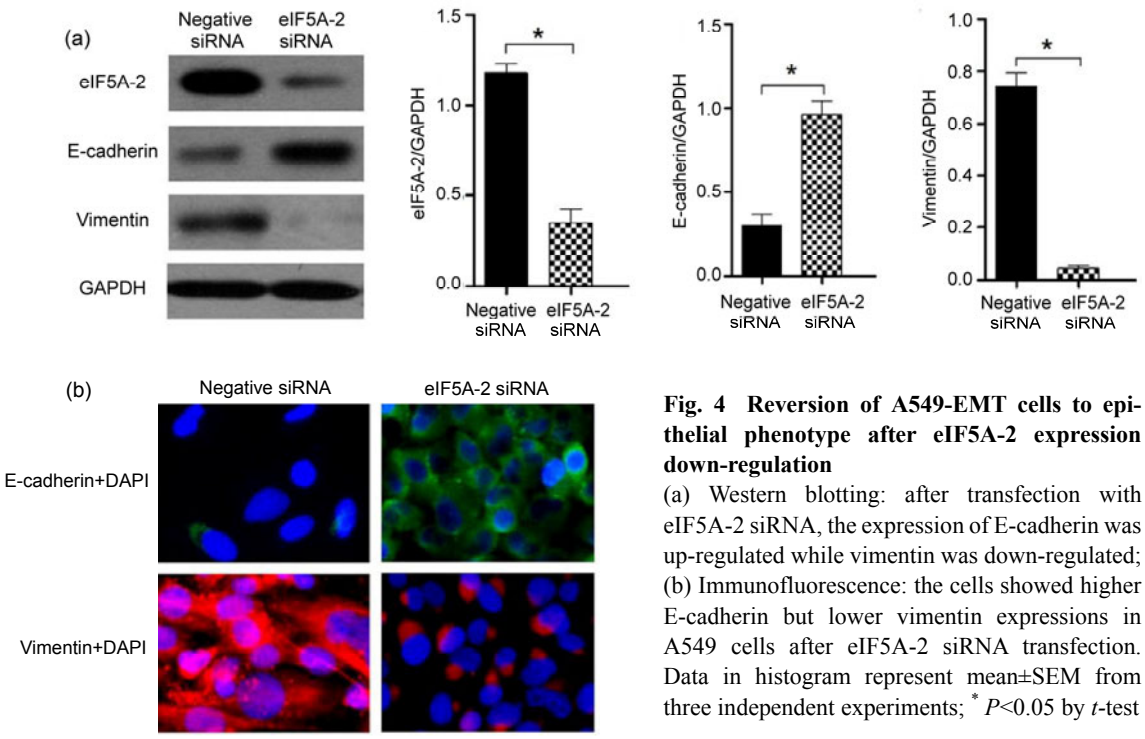

Fig. 4 Reversion of A549-EMT cells to epithelial phenotype after eIF5A-2 expression down-regulation

(a) Western blotting: after transfection with eIF5A-2 siRNA, the expression of E-cadherin was up-regulated while vimentin was down-regulated; (b) Immunofluorescence: the cells showed higher E-cadherin but lower vimentin expressions in A549 cells after eIF5A-2 siRNA transfection. Data in histogram represent mean±SEM from three independent experiments; * $P<0.05$ by t-test

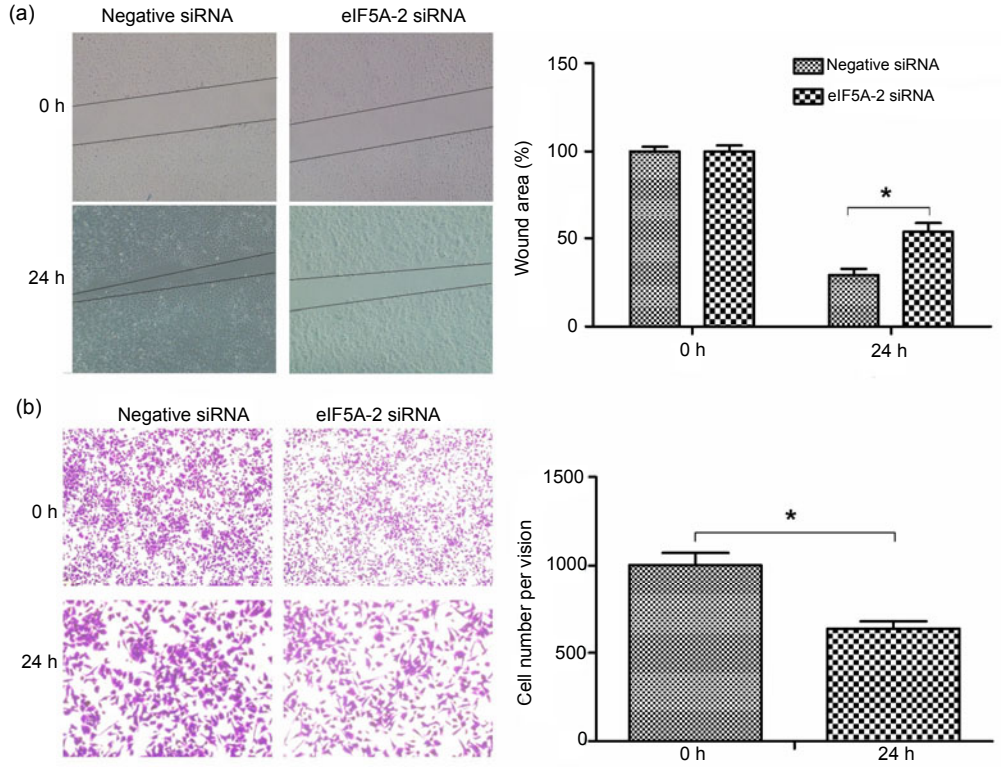

Fig. 5 Weakened migration and invasion capabilities of A549-EMT cells after down-regulation of eIF5A-2 expression

(a) Wound healing assay: fewer cells crossed over the wound edge after transfection with eIF5A-2 siRNA compared with negative siRNA transfection cells; (b) Transwell-matrigel invasion assay: fewer cells invaded through the matrigel after transfection with eIF5A-2 siRNA compared with cells transfected with negative siRNA. Data in histogram represent mean±SEM from three independent experiments; * $P<0.05$ by t-test

metastases in colon cancer (Xie *et al.*, 2008), ovarian cancer (Yang *et al*., 2009), and bladder cancer (Chen *et al.*, 2009). A series of in vitro and in vivo assays were undertaken to characterize the role of eIF5A-2 in the regulation of malignant tumor cell motility and invasiveness, and the results revealed that overexpression of eIF5A-2 could significantly enhance both tumor properties (Tang *et al*., 2010; He *et al.*, 2011). He *et al*. (2011) showed that the expression of eIF5A-2 in normal lung tissue specimens was either absent or low. Furthermore, they found abnormalities in eIF5A-2 in patients with NSCLC, including an association between eIF5A-2 overexpression and the presence of metastases. They suggested that overexpression of eIF5A-2 correlates with local invasion in NSCLC, and might therefore serve as an adverse prognostic marker of survival in stage I disease. Intracellular depletion of eIF5A-2 has also been shown to cause inhibition of cell growth and to induce cell apoptosis (He *et al.*, 2011).

For this reason, an investigation into the relationship between the eIF5A-2 oncogene and NSCLC is essential for an understanding of the mechanisms of tumor recurrence and metastatic disease, and might be helpful for identifying new targets for treating NSCLC.

In this study, we found that overexpression of eIF5A-2 promoted A549 cells to undergo EMT phenotypic changes, which were revealed as up-regulation of the mesenchymal marker vimentin, and down-regulation of the epithelial marker E-cadherin. Following EMT, tumor cells possess enhanced migration and invasion capabilities (Maitah *et al*., 2011), and are less sensitive to chemotherapeutics. Nozawa *et al.* (2006) immunohistochemically examined the expression of epithelial markers in 35 primary lung adenocarcinomas, and found that expression of the E-cadherin/catenin complex was correlated with the differentiation grade of the adenocarcinoma. Other studies found also that E-cadherin loss is relevant, in terms of its association with the development of metastases and a poor prognosis. Low or no expression of E-cadherin is now recognized as an important marker of the occurrence of EMT in malignant tumors, including lung cancer (Thiery, 2002). For this reason, understanding of the relationship between the EMT process and the development of metastases or invasive disease in lung cancer would help in the search for novel EMT targeted therapies (Grünert *et al*., 2003).

Some reports have shown that TGF-β1 can induce A549 NSCLC cells to undergo EMT phenotype changes (Kasai *et al.*, 2005; Kim *et al.*, 2007; Saito *et al.*, 2009). In this study, we induced A549-EMT cells successfully after TGF-β1 stimulation, and these A549-EMT cells became an ideal platform for studying the mechanism of A549 cells EMT phenotype changes and targeted therapy for NSCLC. Our results showed that eIF5A-2 protein expression was higher than that observed in normal A549 parental cells. It has also been shown that blocking the gene expression of eIF5A-2 can inhibit cell growth and induce apoptosis (Guan *et al.*, 2004; Chen *et al.*, 2009; Yang *et al.*, 2009; Tang *et al.*, 2010). In our study, we found that, following gene silencing of eIF5A-2 using eIF5A-2 siRNA, despite exposure to TGF-β1, when compared to A549-EMT cells, all A549 cells maintained their epithelial morphology, with an up-regulated expression of epithelial molecular markers and a down-regulated expression of mesenchymal molecular marker. In addition, the migration and invasion capabilities of A549 cells were clearly less. These results strongly support eIF5A-2 as an upstream factor causing EMT, and suggest that overexpression of EIF5A-2 plays an important role in the invasiveness and metastatic potential of NSCLC, caused by EMT phenotype changes.

Our reuslts demonstrate that overexpression of eIF5A-2 plays an important role in EMT phenotype changes in A549 NSCLC cells, and might promote tumor cell invasion and migration capabilities. This finding not only supports the use of eIF5A-2 as an adverse prognostic marker in patients with NSCLC patients, but also provides a new potential approach for treating NSCLC.

Compliance with ethics guidelines

Guo-dong XU, Xin-bao SHI, Le-bo SUN, Qing-yun ZHOU, Da-wei ZHENG, Huo-shun SHI, Yong-liang CHE, Zi-shan WANG, and Guo-feng SHAO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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