RESEARCH ARTICLE

Early Growth Response Protein-1 Involves in Transforming Growth factor-β1 Induced Epithelial-Mesenchymal Transition and Inhibits Migration of Non-Small-Cell Lung Cancer Cells

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Abstract

The zinc finger transcription factor EGR1 has a role in controlling synaptic plasticity, wound repair, female reproductive capacity, inflammation, growth control, apoptosis and tumor progression. Recent studies mainly focused on its role in growth control and apoptosis, however, little is known about its role in epithelial-mesenchymal transition (EMT). Here, we aim to explore whether EGR 1 is involved in TGF- β 1-induced EMT in non-small-cell lung cancer cells. Transforming growth factor (TGF)- β 1 was utilized to induce EMT in this study. Western blotting, RT-PCR, and transwell chambers were used to identify phenotype changes. Western blotting was also used to observe changes of the expression of EGR 1. The lentivirus-mediated EGR 1 vector was used to increase EGR1 expression. We investigated the change of migration to evaluate the effect of EGR 1 on non-small-cell lung cancer cells migration by transwell chambers. After stimulating with TGF- β 1, almost all A549 cells and Luca 1 cells (Non-small-cell lung cancer primary cells) changed to mesenchymal phenotype and acquired more migration capabilities. These cells also had lower EGR 1 protein expression. Overexpression of EGR 1 gene with EGR 1 vector could decrease tumor cell migration capabilities significantly after adding TGF- β 1. These data showed an important role of EGR 1 in the EMT of non-small-cell lung cancer cells, as well as migration.

Keywords: Non-small-cell lung cancer (NSCLC) - epithelial-mesenchymal transition (EMT) - early growth response

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Introduction

Epithelial-mesenchymal transition (EMT) is now widely accepted as the ability of epithelial cells to undergo mesenchymal transitions during embryogenesis, wound healing and malignant tumor progression (De Craene and Berx, 2013). EMT is characterized by breakdown of cell junctions and loss of epithelial phenotypes, thus contributing to cancer progression and endowing cancer cells with higher ability of migration, invasion and metastasis (Thiery et al., 2009, Liu et al., 2015). Cell adhesion and polarity in epithelia depends on the formation of adherens junctions. E-cadherin is a key determinant during EMT process, providing the physical structure for both cell-cell attachment and the recruitment of signaling complexes (Yu et al., 2014). Several transcription factors have been said to drive EMT, for example, Snail, Slug, ZEB1, ZEB2, E47, and Twist (Haslehurst et al., 2012). EMT can be prompted by various growth factors. Those growth factors include transforming growth factor β (TGF-β) (Serrano-Martinez et al., 2012), hepatocyte growth factor (HGF) (Farrell et al., 2014), members of the epidermal growth factor (EGF) family (Lo et al., 2007), insulin-like growth factor (IGF) (Li et al., 2014), and fibroblast growth factor (FGF) (Acevedo et al., 2007).

Transforming growth factor- β (TGF- β) is a multifunctional cytokine, which regulates cell differentiation, proliferation, motility and apoptosis (McLeod et al., 1990, Portella et al., 1998). TGF- β expression has been studied in various cancers types, including prostate, breast, lung, colorectal, pancreatic, liver, skin cancers, and gliomas (Padua and Massague, 2009). TGF- β has been shown to stimulate angiogenesis (Sanchez-Elsner et al., 2001), evade immune (Gorelik and Flavell, 2001) and epithelial-mesenchymal transition (EMT) (Zhu et al., 2013) in the tumor progression.

The zinc finger transcription factor EGR 1 (Sukhatme et al., 1988), also known as zif268, NGFI-A, Krox24, and TIS8, is induced by many environmental signals including growth factors, hormones, and Hypoxia. Numerous biological roles have been attributed to EGR1, ranging from controlling synaptic plasticity, wound repair, female reproductive capacity, inflammation, coagulation growth control, and apoptosis (Thiel et al., 2010). In the present study, we found TGF- β 1 can inhibit EGR 1 expression of non-small-cell lung cancer cells. EGR 1 overexpression will reduce lung cancer cells migration ability caused by TGF- β 1.

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Li-Na Shan et al Materials and Methods

Cell culture and drug treatment

The primary tumor cells used in this study (designed Luca 1) were categorized as stage IV serious NSCLC that is a disease with poor prognosis (Yan et al., 2013; Lu et al., 2013; Liu et al., 2013; Liu et al., 2013). The human NSCLC cell line A549 was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). All cells were cultured in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA). The cells were maintained at 37°C in 5% CO₂ atmosphere. To induce A549-EMT cells, transforming growth factor (TGF)- β 1 (5ng/ml) (PeproTech, USA) was added to the medium, then, the cells were cultured for 72 hr.

Construction and transfection of lentiviral vectors with EGR 1 overexpression

To further to investigate the function of EGR1, the EGR1 overexpression lentiviral vector (Lenti-EGR 1) was constructed (Shanghai GeneChem Co., Ltd., Shanghai, China). A GFP lentiviral vector was used as negative control (NC). All lentiviral vectors expressed GFP, which enabled us to select stably transfection cells. The day before transfection, A549 cells and Luca1 cells were seeded in 24-well plates at a density of 50 000 cells per well respectively. The lentiviruses were transfected according to the manufacturer's instruction with MOI =30, and stably transfection cells were selected by flow cytometry.

SDS-PAGE and Western blot

To examine the protein level of EGR 1, E-cadherin, Slug and Snail, cells were collected and lysed on ice for 10 min in RIPA Lysis Buffer (Beyotime, Jiangsu, China) with protease inhibitor phenylmethanesulfonyl fluoride (PMSF, Beyotime, Jiangsu, China). 20µg of total protein from each sample was separated on 10% polyacrylamide gels (Beyotime, Jiangsu, China). After electrophoresis, separated proteins were transferred onto polyvinyllidenediflouride (PVDF) membranes (Roche Applied Science). Membranes were then blocked for 1 hr at room temperature with 5% BSA in TBST. The PVDF membranes were, respectively, incubated over night with the rabbit polyclonal anti-EGR-1 (dilution 1:1 000; Cell Signaling Technology, Boston, USA), rabbit monoclonal anti-E-cadherin, anti-Slug, anti-Snail (dilution 1:500; Santa Cruz Biotechnology, CA), and mouse monoclonal anti-GAPDH (dilution 1:1, 000; Beyotime, Jiangsu, China). After washing with TBST, membranes were probed with goat anti-rabbit IgG (dilution 1:5, 000) or goat anti-mouse IgG (dilution 1:5, 000) conjugated with HRP for 1 hr at room temperature. Labeled bands were detected by BeyoECL Plus (Beyotime, Jiangsu, China). Results expressed relative to GAPDH band density used as a loading control.

Real-time PCR

Total RNA was isolated using TRIZOL Reagent (Invitrogen, CA) according to the manufacturer's instructions. For complementary DNA (cDNA) synthesis,

1µg of total RNA was reverse transcribed using a TaKaRa PCR Kit (TaKaRa, Tokyo, Japan) and carried out in triplicate with an ABI 7500 Prism Sequence Detection System (Applied Biosystems, Foster City, CA). The reaction conditions were as follows: 95°C for 30 seconds, followed by 40 cycles of: 95°Cfor 5 seconds, 60°C for 34 seconds. For normalization of all RT-PCR data, GAPDH expression was used as a reference gene. Primers used in real-time PCR were as follows: EGR 1 sense 5'-CAGCACCTTCAACCCTCAG-3', antisense 5'-CACAAGGTGTTGCCACTGTT- 3'; E-cadherin sense 5'-GAGTGCCAACTGGACCATTCAGTA-3', antisense 5'-AGTCACCCACCTCTAAGGCCATC-3'; Slug sense 5'-AGATGAGCATTGGCAGCGAG-3', antisense 5'-CAGGAGAAAATGCCTTTGGA-3'; Snail sense 5'-TCGGAAGCCTAACTACAGCGA-3', antisense 5'-AGATGAGCATTGGCAGCGAG-3'; GAPDH sense 5'-GGTCTCCTCTGACTTCAACA-3', antisense 5'-AGCCAAATTCGTTGTCATAC-3'.Relative transcript abundance of E-cadherin, Slug, Snail, were expressed in Δ Ct values (Δ Ct = Ct target -Ct reference). Relative fold changes in transcript levels compared to basal levels was calculated as $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct$ treatment- Δ Ct basal).

Cell migration assay

In migration assay, 3×10^4 cells were suspended in a 200 μ l serum-free medium and then seeded in the upper chambers of a transwell plate (8.0 μ m, Millipore, Billerica, MA), while the lower chambers were filled with RPMI-1640 containing 15% FBS. The plates were incubated for 24 hours at 37°C in 5% CO₂. Cells that did not migrate through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were examined and counted under a microscope. Each experiment was repeated at least three times.

Results

TGF- β 1 induces Epithelial-to-mesenchymal transition of A549 and Luca 1 cells in vitro

A549 cells and Luca 1 cells cultured in the absence of TGF-B1 maintained the classic cobblestone epithelial morphology and growth pattern, but after treatment of 5 ng/mL TGF- β 1 for 72 hr, the cells showed a more fibroblast-like morphology (Figure 1A). Following stimulation by TGF- β 1 (5ng/ml) for 72 hr, we tested three typical molecular markers for A549 cells and Luca 1 cells through RT-PCR and western blotting. The RT-PCR results showed in two pairs of NSCLC cell lines (A549 and Luca 1) treated with or without TGF- β 1, E-cadherin expression level was effectively reduced to 10±4 % in A549 cells and 15±3% in Luca 1 cells treated with TGF-β1 compared with untreated group. Slug expression level was increased to almost 4-folds in A549 cells and almost 4.1-fold in Luca 1 cells in TGF- β 1-treated group compared with the untreated group. Snail also increased almost 1.6-fold in A549-EMT cells and almost 4.2-fold in Luca 1-EMT cells by untreated group respectively (Figure 1B). Western blotting results showed that E-cadherin was markedly down-regulated, and the markers, Slug and Snail, were

markedly up-regulated compared to the untreated A549 parental cells. Luca 1 cells also showed similar results (Figure 1C).

Migration and invasion capabilities of A549 cells and Luca 1 cells were enhanced after stimulation by TGF- β 1

Studies have demonstrated that tumor cells show enhanced migration and invasion capabilities following EMT. In this study, we compared the migration capability of A549 cells and Luca 1 cells before and after exposure to TGF- β 1 by transwell chambers. After exposure to TGF- β 1 (5ng/ml) for 72 hr, 3× 10⁴ cells (A549, A549-EMT, Luca1, Luca1-EMT) were suspended in a 200 μ 1 serumfree medium and then seeded in the upper chambers of a transwell plate. As shown in Figure 2, we found that the A549-EMT cells (399 ±18) exhibited increased migration



Figure 1. EMT Phenotype Changes on Exposure to TGF- β 1 (5 ng/ml) for 72hr. (A) Morphologic changes in A549 cells and Luca1 cells treated with TGF- β 1. (B) The mRNA level of E-cadherin was down-regulated, and the mRNA level of Slug and Snail was up-regulated both in two pairs of lung cancer cell lines (A549 and Luca1). (C) Western blotting assay showed the E-cadherin expression was down-regulated, and Snail and Slug expression was up-regulated in two pairs of lung cancer cell lines (A549 and Luca1).Data in histogram represent mean±SEM from three independent experiments; **P<0.01 by t-test

in comparison with A549 NC (200±13) (P<0.05) and Luca 1-EMT cells (296±16) also showed increased migration in comparison with Luca 1 NC cells (142±17) (P<0.05). The results show that the A549-EMT cells have a greater capacity to migrate after exposure to TGF- β 1 than the A549 parental cells. Luca1-EMTcells also show greater migration capacity than Luca1 parental cells. Suggesting that exposure to TGF- β 1 can enhance the migration capability of Non-small-cell lung cancer cells.



Figure 2. Enhanced Migration Capabilities of A549 Cells and Luca 1 Cells after Stimulation by TGF-β1 (5 ng/ml) for 72 h. There was highly cell migratory ability in A549 cells and Luca 1 cells after treating with TGF-β1. Data in histogram represents mean±SEM from three independent experiments; **P<0.01 by t-test



Figure 3. The Expression of EGR 1 Protein in A549 Cells and Luca1 Cells after Stimulation by TGF- β 1. (A) RT-PCR showed that in two pairs of lung cancer cell lines (A549 and Luca1) treated with or without TGF-b1, the EGR 1 expression was down-regulated. (B) The protein level of EGR 1 was down-regulated. ***P*<0.01 by t-test

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Cells had lower expression of the EGR-1 protein after TGF- β 1 induction

To further to investigate how TGF- β 1 induces A549 cell EMT, we focused on the effects of the EGR 1 in this transition, as EGR 1 might have some connection with EMT that then leads to tumor migration and the development of metastases. We detected EGR 1 RNA expression by RT-PCR and EGR 1 protein expression by Western blotting at 3 hr after adding TGF- β 1. The results showed, compared to normal A549 cells, EGR1 protein expression was markedly down-regulated in the A549-EMT (Figure 3 A, B). Luca1-EMT cells also showed the similar result than normal Luca1 cells (Figure 3 A, B). These results show TGF- β 1 can inhibit EGR 1 expression.

Addition of EGR 1 expression could decrease tumor cell migration capability

To further to ascertain the role of EGR 1, a lentivirusbased delivery system was utilized to transfer EGR 1 vector into A549 cells and Luca 1 cells. To test the efficiency of the EGR 1 vector, EGR 1 expression level was tested by RT- PCR and Western blotting assays. Western blot results showed the EGR 1 expression was evidently increased by EGR 1 vector compared with the NC in both A549 group and Luca 1 group (Figure 4B).



Figure 4. Weakened Migration Capabilities of A549-EMT Cells and Luca 1-EMT cells after Overexpression of EGR 1 Expression. (A) The mRNA level of EGR 1 in two pairs of in two pairs of lung cancer cell lines (A549 and Luca1) was up-regulated after adding of Lenti-EGR 1. (B) Western blotting results showed the protein level of EGR 1 was upregulated after transfection Lenti-EGR 1. (C) There was less cell migratory ability in A549-EMT cells and Luca 1-EMT cells after EGR 1 up-regulation. Data in histogram represent mean±SEM from three independent experiments;*P<0.05, **P<0.01 by t-test

Similar trend was found in mRNA level (Figure 4A).

We have shown that increasing EGR 1 expression of A549 cells and Luca 1 cells. Then we used transwellmigration assay to detect if EGR 10verexpression could affect the EMT. Transwell-migration assays showed that the number of cells in the high power lens of each field of vision in EGR 1-A549-EMT cells (284±6) was less than which in A549-EMT (NC) cells (399 ± 10) (Figure 4C). The number of cells was only 202±10 in EGR 1-Luca 1-EMT cells, which were significantly less than that observed in the negative EGR 1 group in Luca 1 group (307 ± 20) (P<0.05) (Figure 4C). These results suggest that following over expression of EGR 1 in A549-EMT cells and Luca 1-EMT cells, cell behavior can change markedly. All these results suggest that EGR1 is associated with EMT, and can decrease metastatic capabilities of non-small-cell lung cancer cells.

Discussion

The developmental program of epithelial to mesenchymal transition (EMT) can be activated in tumor cells by TGF- β . EMT leads to loss of cell adhesion and increases motility of cells EMT can be detected by down regulation of epithelial makers such as E-cadherin and gain of mesenchymal makers such as vimentin and fibronectin. A large number of gene expressions are changed during this process. In our study, we found inhibition expression of EGR 1 during the EMT, and showed a rapid process.

The transcription factor Egr-1 is an immediateearly response gene that is rapidly induced by various growth factors, cytokines, DNA-damaging agents and so on. In a report by Mingui FU (Fu et al., 2003), it was shown that TGF- β rapidly and transiently induced early growth response factor-1 (Egr-1) expression through the mitogenactivated protein kinase extracellular signalregulated kinase kinase 1 (MEK1)/ERK-mediated pathway in the development of vascular diseases.

The function of EGR1 in tumor growth, development and apoptosis has been extensively investigated. The role of Egr-1 in tumor development might depend to a large extent on the tissue type. Egr-1 can function as a tumor suppressor in certain types of cancer. HUANG et al. (1997) found loss of Egr-1 expression correlates with the development of breast cancer. Huihua et al. (2014) showed that EGR1 arrested cell mobility, inhibited migration, and induced apoptosis in in non-small-cell lung carcinoma (NSCLC). Moon et al. (2007) found EGCG was strong inducer of EGR-1 expression and mediated EGR-1 nuclear translocation via ERK signaling pathway in A549 pulmonary epithelial cells. Induced EGR-1 then stimulated the induction of mPGES-1 gene expression and this effect mechanistically can be linked to the pharmacological or toxicological actions after human exposure to green tea catechins. In contrast, Egr-1 is highly expressed and plays an essential role in tumor growth and survival in prostate cancer (Virolle et al., 2003). In the study of VERONIQUE BARON et al. (2003), they found treatment with Egr-1 antisense oligonucleotides delayed the occurrence of prostate tumors in TRAMP mice. Soon Young Shin (Shin et al., 2010) found stable silencing of Egr-1 by siRNA

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strongly attenuated the invasiveness of HeLa cervix carcinoma cells.

Egr-1 also showed a different function during EMT. Sun et al. (2014) found hypoxia treatment enhanced the mRNA and protein levels of Egr-1 in HK-2 cells, which was accompanied by a reduced expression of the epithelial marker E-cadherin and an enhanced expression of the mesenchymal marker Fsp-1. Down-regulation of Egr-1 with siRNA reversed hypoxia-induced EMT. EGR1 was previously described to be down-regulated in many breast carcinoma tissues (LIU et al., 2007), while it was upregulated together with SNAI1 and FOS in the highly invasive inflammatory breast carcinoma (Parra et al., 2013). Vetter et al. (2009) found EGR1 exhibited a transient, biphasic expression behavior which was confirmed by real-time PCR. EGR1started to be upregulated 4h after SNAI1 induction, and was repressed after 24h.

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