

## Original Article

# Interleukin-17-induced EMT promotes lung cancer cell migration and invasion via NF- $\kappa$ B/ZEB1 signal pathway

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**Abstract:** Inflammatory cytokine interleukin-17 (IL-17) has been associated with the risk of progressive cancers including lung cancer. However, it remains unclear how IL-17 may contribute to the invasion and development of these inflammation-associated malignancies. Here we aimed to investigate the role of IL-17 in lung cancer cell development. Epithelial-mesenchymal transition (EMT) has been recently proposed as a developmental process which plays an important role in cancer progression and metastases. Here we show that IL-17 might promote EMT in lung cancer cells by inducing the transcriptional repressor ZEB1. Exposure to IL-17 upregulated the signature EMT phenotypic markers vimentin and E-cadherin in lung cancer cells, and compared with controls, increased cell migration was observed in IL-17-treated lung cancer cells. ZEB1 mRNA and protein expression was induced by IL-17, and IL-17 stimulated nuclear localization of phosphorylated ZEB1. Conversely, suppressing ZEB1 expression by ZEB1 siRNA abrogated IL-17-stimulated vimentin expression and cell migration. Moreover, the phosphorylation of I $\kappa$ B $\alpha$  was required for IL-17-induced expression of ZEB1, suggesting the involvement of canonical NF- $\kappa$ B signaling. To check this hypothesis, we used IKK inhibitor BAY 11-7028 to block NF- $\kappa$ B activity. We found that BAY 11-7028 abrogated IL-17-induced ZEB1 expression, cell migration, and EMT, thus confirming that NF- $\kappa$ B is required for IL-17 to induce these aggressive phenotypes in lung cancer cells. Taken together, our data support the idea that IL-17-induced EMT promotes lung cancer cell migration and invasion via NF- $\kappa$ B-mediated upregulation of ZEB1. This study reveals a new signaling axis through which the tumor microenvironment causes ZEB1 expression to promote cancer metastasis. We suggest that targeting IL-17-induced ZEB1 expression may offer an effective therapeutic strategy for lung cancer treatment.

**Keywords:** Interleukin-17, epithelial-mesenchymal transitions, NF- $\kappa$ B, ZEB1, lung cancer

## Introduction

According to data from the World Health Organization, lung cancer accounts for 1.6 million deaths each year, or 19.5% of the total cancer mortality worldwide. Even with combined treatments including surgery, chemotherapy, radiotherapy, and targeted therapy, the outcomes for patients with lung cancer remain poor with an overall 5-year survival rate around 15%, largely due to development of metastases [1]. Therefore, a better understanding of the mechanisms that contribute to invasion and metastasis in lung cancer is crucial for the development of novel therapeutic strategies to reduce mortality caused by this malignancy. It is widely accepted that the network of cytokines and infiltrating immune cells in the tumor

microenvironment controls the pattern of tumor invasiveness; however, very few studies have demonstrated the unique behavior of specific cytokines in the metastasis of tumors [2-6].

Interleukin-17 (IL-17) is a pleiotropic cytokine that plays a vital role in many chronic inflammatory diseases. Recent studies have shown that IL-17 plays a primary role in the pathophysiology of cancer [7-9]. In lung cancer, tumor tissue exhibits high expression levels of IL-17 compared with matched normal lung tissue samples, and increased levels of IL-17 correlate with more advanced tumor grades. Furthermore, elevated serum IL-17 levels correlate with advanced lung cancer stage, increased number of metastatic sites, and poor survival in patients with lung cancer [10-12]. However, the direct

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effect of IL-17 on the metastases of lung cancer cells still remains largely unknown. Thus, in this study, we attempted to elucidate the possible role and associated molecular mechanism of IL-17 in the lung cancer cell invasion and metastasis *in vitro*.

Epithelial-mesenchymal transition (EMT), a complex reprogramming process of epithelial cells, plays an essential role in tumor invasion and metastasis [13, 14]. The representative characteristics of EMT include the loss of epithelial markers (E-cadherin, and  $\alpha$ - and  $\gamma$ -catenin) and the gain of mesenchymal cell markers (fibronectin, vimentin, and N-cadherin), leading to the acquisition of migratory and invasive properties [15-17].

Recent studies show that EMT is controlled by a group of transcriptional repressors, including ZEB1, Twist1, Snail, and Slug. ZEB1, known as a master regulator of morphogenesis, induces EMT to facilitate lung tumor metastasis [18, 19]. Upon activation, these repressors recruit histone deacetylases to the E-box elements of the E-cadherin promoter, resulting in the transcriptional silencing of E-cadherin expression. In lung cancer, ZEB1 appears to be a major factor in the EMT process. In lung cancer cell lines, it was previously found that the loss of E-cadherin was inversely and specifically correlated with ZEB1 mRNA expression [18]. These findings were recently confirmed, and it was further demonstrated that ZEB1 positively influences the anchorage-independent growth of these cells [20]. NF- $\kappa$ B signaling also played an important role in this process [21-26]. Both EMT and ZEB1 expression can be induced via the NF- $\kappa$ B signaling pathway [27, 28], and IL-17 is the most important element in the activation of this signaling pathway [29, 30].

Therefore, we hypothesized that IL-17 may stimulate EMT in lung cancer cells via the NF- $\kappa$ B/ZEB1 signaling pathway thus promoting cancer progression and metastases. The aim of our study was to test this hypothesis *in vitro* by determining if IL-17 stimulates the key phenotypic and functional features of EMT associated with cancer progression in lung cancer cell lines.

### Materials and methods

#### Cell culture

The human lung cancer cell line A549 was obtained from the American Type Culture

Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) containing 25 mM HEPES buffer supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) at 37°C with 5% CO<sub>2</sub> in a humidified incubator. Cells were routinely passaged and, when at logarithmic growth phase, used for the study.

#### Western blot analysis

Cells were washed twice with cold PBS and lysed on ice in radioimmunoprecipitation (RIPA) assay buffer (Beyotime Institute of Biotechnology, China). Cellular lysates were clarified by centrifugation, and protein concentrations of the lysates were determined using a bovine serum albumin standard line. Equal amounts of protein were boiled at 100°C for 10 min and chilled on ice, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and then electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat dry milk (w/v) in Tris-buffered saline (pH 8.0) with 0.1% Tween-20 (TBS-T) and then immunoblotted overnight at 4°C with rabbit anti-human-NF- $\kappa$ B (dilution 1:500, Cell Signaling Technology (CST), MA, USA), mouse anti-human-E-cadherin, mouse anti-human-vimentin, rabbit anti-human-Twist, rabbit anti-human-ZEB1, and rabbit anti-human-Snail (dilution 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-human-Slug (dilution 1:1000, Abcam, Cambridge, MA, USA), or mouse anti-GADPH antibody (dilution 1:5000, Sigma), followed by their respective horseradish peroxidase-conjugated secondary antibodies. After extensive washing, the bands were revealed by an ECL Plus chemiluminescence kit (Millipore, Bedford, MA, USA).

#### Immunofluorescence staining

After designated treatment, A549 cells were fixed with 4% paraformaldehyde for 10 min, permeabilized in 0.5% Triton X-100 for 10 min, and incubated in PBS and 10% horse serum blocking solution for 1 h. Fixed cells were incubated for 2 h with mouse anti-human-E-cadherin, mouse anti-human-vimentin, rabbit anti-human-NF- $\kappa$ B (dilution 1:200, Cell Signaling Technology (CST), MA, USA), and rabbit anti-human-ZEB1 (dilution 1:500, Santa Cruz

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**Table 1.** Primers used for qRT-PCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Length
E-cadherin	TTGCTCACATTTCCCAACTCCTC	CACCTTCAGCCATCCTGTTTCTC	234 bp
Vimentin	GCTGAATGACCGCTTCGCCAACT	AGCTCCCGCATCTCCTCCTCGTA	144 bp
NF-κB	TGGTGAAGACCTTGCTGCTAAATGC	ACTGGGTGAGGTTGTCTGTCGGTA	221 bp
ZEB1	ACTGGGTGAGGTTGTCTGTCGGTA	AAAGGAAGACTGATGGCTGAAAT	143 bp
Snail	TCTAGGCCCTGGCTGCTACAA	ACATCTGAGTGGGTCTGGAGGTG	131 bp
Slug	ATGCATATTCGGACCCACACATTAC	AGATTTGACCTGTCTGCAAATGCTC	158 bp
Twist	GGCGGCCAGGTACATCGACTT	GCTAGTGGGACGCGGACAT	157 bp
GAPDH	AGAAGGCTGGGGCTCATTG	AGGGCCATCCACAGTCTTC	238 bp

Biotechnology, Santa Cruz, CA, USA) in 5% horse serum. Cells were washed and incubated with goat anti-mouse FITC (green) or goat anti-rabbit RBITC (red) IgG antibody (ZSGB-BIO Inc., Beijing, China) diluted 1:100 in blocking buffer for 1 h. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 3-5 min. Cells were examined with a fluorescent microscope equipped with narrow band-pass excitation filters to individually select for green, red, and blue fluorescence. Cells were observed through a Canon PowerShot A640 camera mounted on a fluorescent microscope (Nikon, Japan). Experiments were repeated thrice.

### *Transfection with small interfering RNA (siRNA) targeting ZEB1 subunit*

The siRNA targeting ZEB1 mRNA was synthesized by Genechem Co, Ltd (Shanghai, China). The siRNA sequence for ZEB1 targeting was 5'-GCCCUAUCCUUUACGUCA-3'. Negative control siRNA consisted of a scrambled sequence that does not lead to the specific degradation of any known cellular mRNA. At 80-90% confluency, the cells were treated with siRNA using Lipofectamine® 2000 (Invitrogen) according to the recommended protocol.

### *Quantitative real-time polymerase chain reaction*

At 48 h after siRNA transfection, the cells were lysed for RNA isolation. Total RNA was extracted by using Trizol. A SuperScript™ III Platinum® SYBR® Green one-step quantitative real-time polymerase chain reaction (qRT-PCR) kit (Invitrogen, Carlsbad, CA, USA) was used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control gene for all analyses. Primer sequences are given in **Table 1**. Primer specificity was assessed from mono-

phase dissociation curves, and all primers had similar efficiency (data not shown). The threshold cycle ( $C_t$ ) for the endogenous control GAPDH mRNA and target signals were determined, and relative RNA quantifi-

cation was calculated using the comparative  $2^{-\Delta\Delta C_t}$  method, where  $\Delta\Delta C_t = (C_t^{\text{Target}} - C_t^{\text{GAPDH}}) - (C_t^{\text{Control}} - C_t^{\text{GAPDH}})$ . All reactions were performed in duplicate.

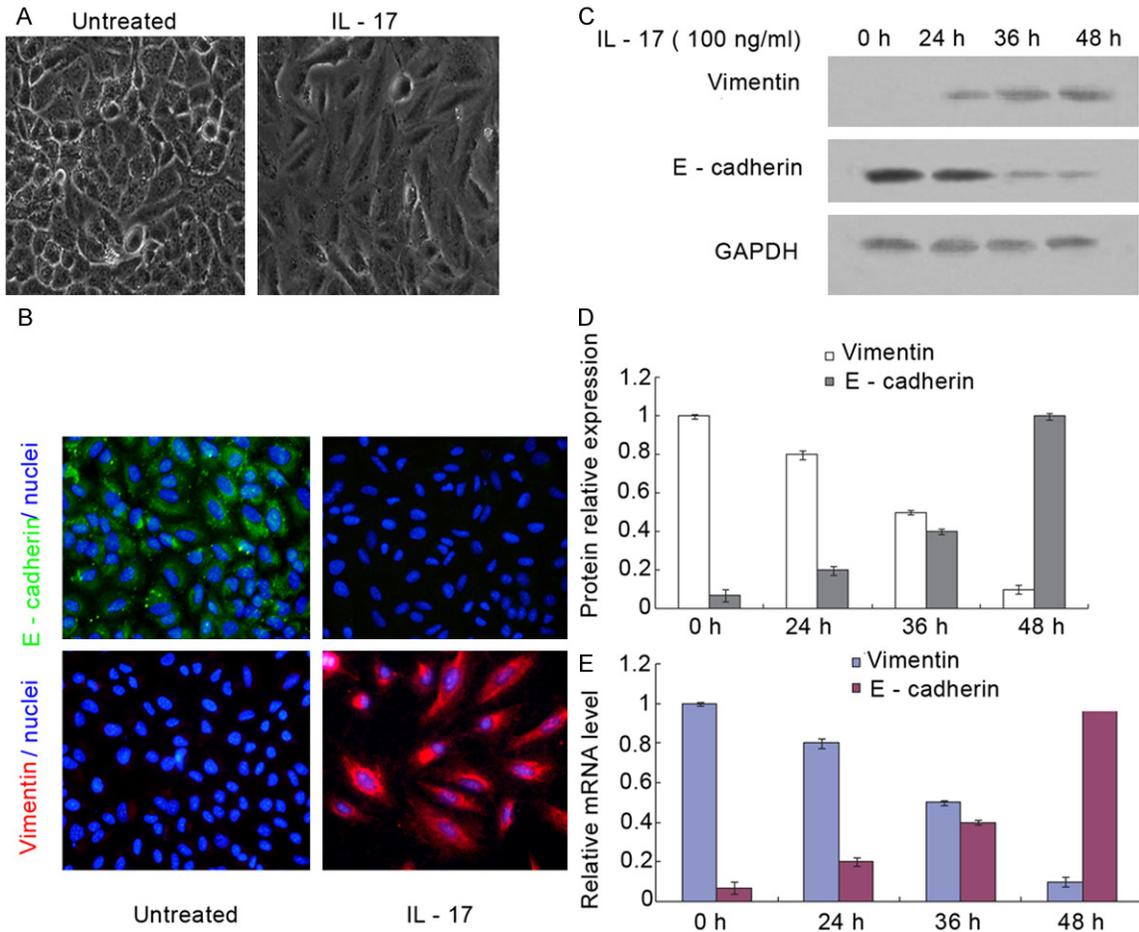
### *Wound healing assay*

Cells were seeded in six-well plates at a density of  $1 \times 10^5$  cells per well and grown to 80-90% confluence. After removal of the culture medium, the cell monolayers were mechanically scraped with a sterile 200- $\mu$ l pipette tip to create a denuded area of constant width, cell debris was removed by washing the monolayer twice with serum-free medium, and the cells were cultured for additional 24 h. The wound closure was monitored and photographed under a microscope to assess the migration ability of the cells. Results were expressed as a migration index, that is, the distance migrated by the cell monolayer to close the wounded area during this time period.

### *In vitro Matrigel™ invasion assay*

*In vitro* migration assays were performed in 24-well transwell chambers (Costar, Cambridge, MA, USA) containing polycarbonate filters with 8- $\mu$ m pores coated with Matrigel™ (1 mg/ml, BD Sciences, San Jose, CA, USA). Briefly,  $5 \times 10^4$  cells in 500  $\mu$ l of serum-free medium were seeded into the upper chamber, and the lower chamber was filled with 750  $\mu$ l of medium containing 10% FBS as a chemoattractant. After a 24 h incubation, the non-invasive cells were removed with a cotton swab, and cells that had migrated through the membrane and stuck to the lower surface of the membrane were fixed with methanol and stained with hematoxylin. Finally, the cell numbers were counted and averaged in six random fields at a magnification of 100 $\times$ .

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**Figure 1.** IL-17 induces EMT phenotype in lung cancer cells. A. Phase contrast analysis (original magnification,  $\times 100$ ) of morphological changes detected in lung cancer cells with or without IL-17 treatment for 48 h. B. Immunofluorescence staining for E-cadherin and vimentin in lung cancer cells under IL-17 conditions for 48 h. Green color represents E-cadherin staining, and red color shows vimentin staining. Blue signal represent nuclear DNA staining by DAPI. C. Western blot analysis of epithelial marker (E-cadherin) and mesenchymal markers (vimentin) in A549 cells with or without IL-17 treatment for 0, 24, 36 and 48 h. Representative blots from three independent experiments are shown. D. The histogram showing the average volume density corrected for the loading control (GAPDH).  $**p < 0.01$ . E. qRT-PCR for mRNA of epithelial marker E-cadherin and mesenchymal marker vimentin mRNA levels. Relative expression was obtained using the  $2^{-\Delta\Delta Ct}$  method after normalization to GAPDH. Histogram shows mean and SEM of mRNA expression of E-cadherin and vimentin in three separate experiments.

### Statistical analysis

The results were expressed as MEAN  $\pm$  SEM (standard error of the mean). Statistical analysis of data and individual differences among groups were analyzed by using the t-test by Sigma Plot 9.0. A value of  $P < 0.05$  was considered a significant difference and  $P < 0.01$  was considered an extremely significant difference.

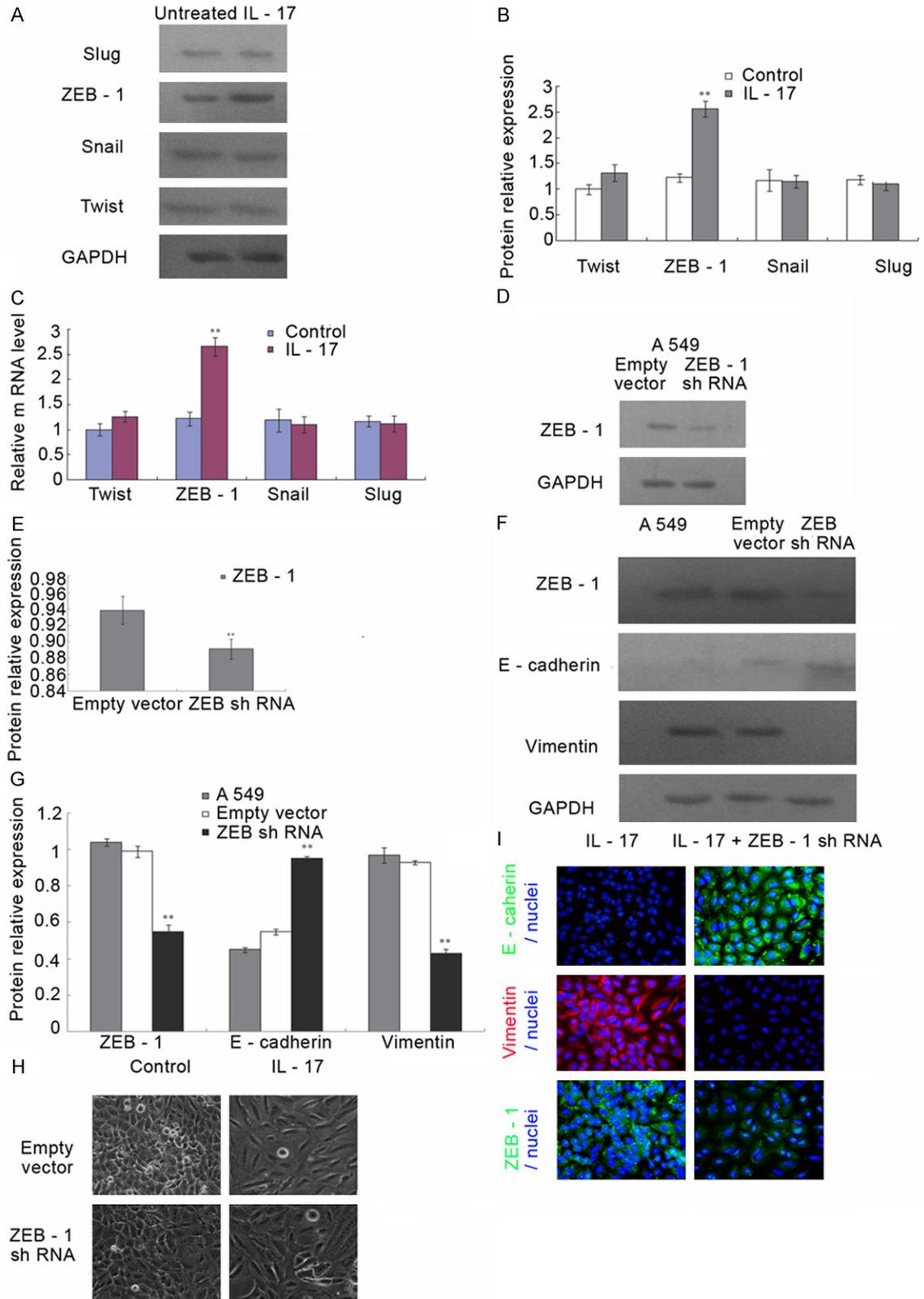
### Results

#### IL-17 induced EMT in A549

EMT is thought to be a key mechanism by which tumor cells acquire their migratory and invasive

capabilities. To determine whether EMT is involved in IL-17-induced migration and invasion, we first observed morphological transformation of cells *in vitro*. As shown in **Figure 1A**, IL-17-untreated A549 cells kept a typical epithelial phenotype in a monolayer culture, characterized by a cobblestone-like appearance and tight cell-cell junction. In contrast, IL-17-treated A549 cells exhibited a spindle-like morphology and the loss of cell-cell contacts, suggesting that overexpression of IL-17 induces EMT-like transformation in lung cancer cells. To corroborate whether this morphological transformation represents EMT, we analyzed the levels of several EMT markers by immunofluores-

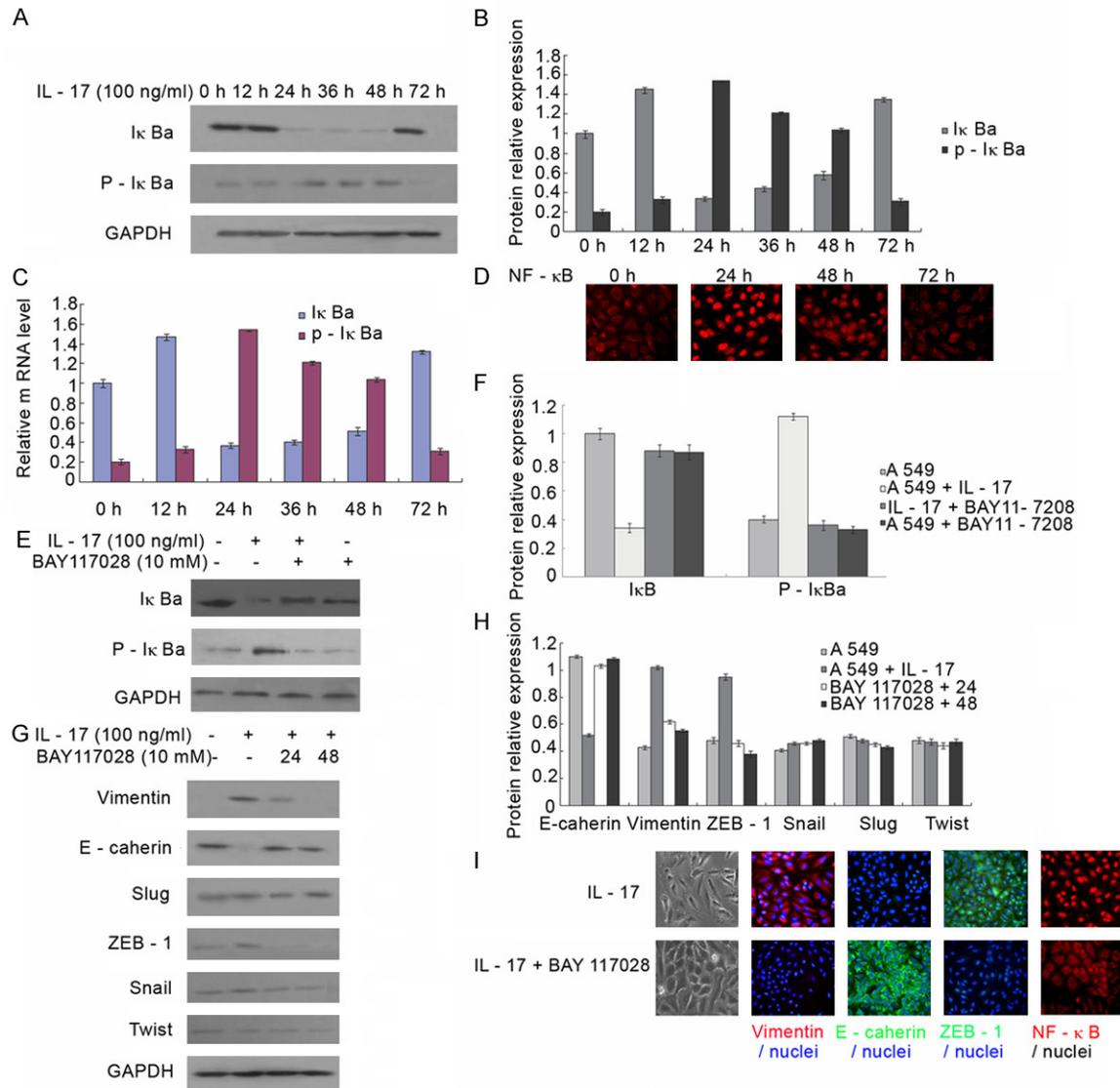
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**Figure 2.** IL-17 induced EMT via ZEB1 activation in lung cancer cells. A. Western blot analysis of baseline expression of EMT-mediating transcription factors Slug, ZEB1, Snail, and Twist in lung cancer cells with or without IL-17 treatment for 48 h. B. The histogram shows the average volume density corrected for the loading control (GAPDH).

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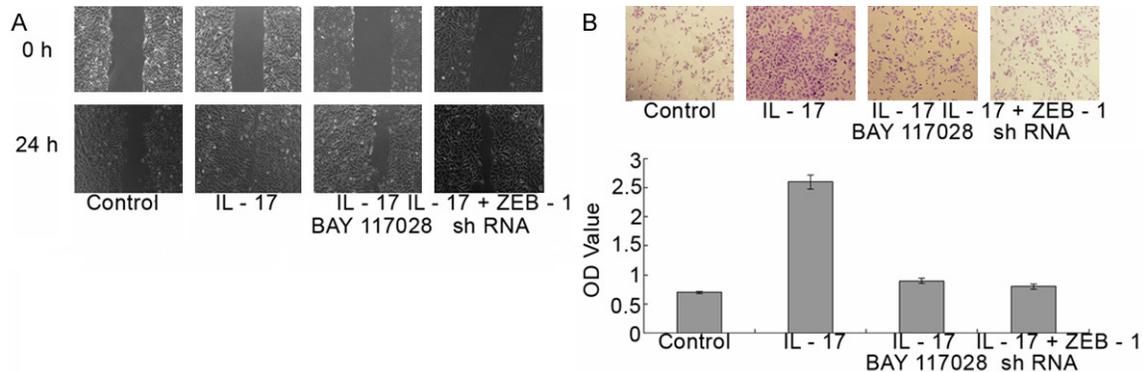
\*\*p < 0.01. C. qRT-PCR for EMT-mediating transcription factor mRNA levels. Relative expression was obtained by using the  $2^{-\Delta\Delta Ct}$  method after normalization to GAPDH. Histogram shows mean and SEM of mRNA expression of Slug, ZEB1, Snail, and Twist in three separate experiments. D. Western blot analysis. Effects of the ZEB1 siRNA on ZEB1 expression in A549 cells under IL-17 treatment conditions for 48 h. E. The histogram shows the average volume density corrected for the loading control (GAPDH). \*\*p < 0.01. F. Western blot analysis. Effects of the ZEB1 siRNA on E-cadherin and vimentin expressions in A549 cells under IL-17 treatment conditions for 48 h. G. The histogram shows the average volume density corrected for the loading control (GAPDH). \*\*p < 0.01. H. Bright field microscopic image of shRNA ZEB1 and empty vector cells (control cells) showing a clear change in cell morphology. I. Immunofluorescence analysis confirmed reduced expression levels of vimentin and increased expression of E-cadherin, as well as nuclear localization of ZEB1 in shRNA ZEB1 cells compared to that of control cells under IL-17 treatment conditions for 48 h.



**Figure 3.** IL-17-induced EMT via ZEB1 depends on NF- $\kappa$ B activation in lung cancer cells. A. Western blot analysis of NF- $\kappa$ B expression in A549 cells treated with IL-17 for 0, 12, 24, 36, 48 and 72 h. B. The histogram shows the average volume density corrected for the loading control (GAPDH). \*\*p < 0.01. C. qRT-PCR for I $\kappa$ B and P-I $\kappa$ B mRNA levels in A549 cells treated with IL-17 for 0, 12, 24, 36, 48 and 72 h. Relative expression was obtained using the  $2^{-\Delta\Delta Ct}$  method after normalization to GAPDH. Histogram shows mean and SEM of mRNA expression of I $\kappa$ B and P-I $\kappa$ B in three separate experiments. D. Confocal immunofluorescence of NF- $\kappa$ B expression and localization in cells treated with IL-17 for 0, 24, 48 and 72 h. E. Western blot analysis. NF- $\kappa$ B expression in IL-17-treated and untreated A549 cells incubated in the presence or absence of NF- $\kappa$ B inhibitor BAY 11-7028 for 48 h. F. The histogram shows the average volume density corrected for the loading control (GAPDH). \*\*p < 0.01. G. Western blot analysis. Express-

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sion of E-cadherin, vimentin, Slug, ZEB1, Snail, and Twist in IL-17-treated and untreated A549 cells incubated in the presence or absence of IKK inhibitor BAY 11-7028 for 24-48 h. (H) The histogram shows the average volume density corrected for the loading control (GAPDH).  $**p < 0.01$ . (I) Immunofluorescence analysis confirmed reduced expression of vimentin and increased expression of E-cadherin, as well as nuclear localization of ZEB1 and NF- $\kappa$ B in the IL-17-treated A549 cells incubated with or without IKK inhibitor BAY 11-7028 for 48 h.



**Figure 4.** IL-17 promotes migration and invasion of lung cancer cells A549 *in vitro*. A. Each of the stable cell monolayers was scratched by a sterile 200- $\mu$ l pipette tip and cell images were taken at the indicated time points. B. Transwell migration assays of the A549 cells. The same number of cells was seeded in each transwell. After 24 h, the number of cells that had migrated to the lower chamber was counted. Values are expressed as the mean  $\pm$  SEM of three independent experiments.  $**p < 0.01$  vs. control cells. Scale bars = 50  $\mu$ m.

cence staining and Western blot analysis. As shown in **Figure 1B** and **1C**, IL-17 treatment diminished the expression of epithelial marker E-cadherin and increased the protein amounts of mesenchymal vimentin in A549 cells ( $P < 0.01$ ).

### IL-17 induced EMT via ZEB1 activation in A549

During EMT, the specific transcriptional repressor, ZEB1, is known to repress transcription of E-cadherin and thus promote the loss of the cell-cell adhesion [31-33]. Hence, we examined the expression of transcriptional repressors in IL-17-treated A549 cells and found that increased levels of IL-17 upregulated EMT transcription factor, ZEB1 (**Figure 2A-C**;  $P < 0.01$ ). Moreover, we investigated whether knockdown of ZEB1 expression with siRNA would prevent IL-17-induced EMT (vimentin expression). A549 cells were pretreated (48 h) with either an irrelevant siRNA control or siRNA specific for ZEB1 (in 100 nM final concentrations each), as described by Sánchez-Tilló and co-authors [34]. After pre-treatment, the cells were treated with IL-17 (100 ng/ml) for 48 h. Western blots and immunofluorescence were used to assess ZEB1 and vimentin protein expression. As seen in **Figure 2F-I**, treatment with IL-17 caused an increase in vimentin expression in both media-

treated and control siRNA-treated cells ( $p < 0.01$ ). We found that knockdown of ZEB1 expression inhibited IL-17-induced vimentin expression, as well as E-cadherin decrease in A549 cells (**Figure 2F**).

Thus, our results strongly suggest that IL-17 induces EMT in A549 lung cancer cells through the upregulation of ZEB1, which is accompanied by the loss of E-cadherin, a marker of EMT, and the gain of mesenchymal markers, including vimentin (**Figure 2F, 2I**).

### IL-17 activated NF- $\kappa$ B activity in A549

Some studies have previously shown that IL-17 activates NF- $\kappa$ B [35, 36]. Thus, we thought to determine whether the EMT observed in A549 under IL-17 conditions was attributable to increased NF- $\kappa$ B activity. A549 cells were incubated with or without IL-17 for different periods of time. At 24 h, 36 h and 48 h, we observed increased p-I $\kappa$ B $\alpha$  in IL-17-treated A549 cells compared with IL-17-untreated cells, as determined by Western blot analysis and immunofluorescence staining. Additionally, we demonstrated that activation of I $\kappa$ B $\alpha$  was time-dependent, increasing significantly after 24 hours of IL-17 stimulation and reaching maximal level after 36 to 48 hours (**Figure 3A, 3B**).

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### *Inhibition of NF- $\kappa$ B activity reverses IL-17-induced EMT in A549*

Once we established that IL-17 treatment resulted in elevated NF- $\kappa$ B activity in A549 cells, we investigated the potential for inhibition of NF- $\kappa$ B to attenuate the mesenchymal characteristics of IL-17-treated A549 cells. We used a commercially available IKK inhibitor, BAY 11-7028, to pharmacologically block NF- $\kappa$ B activity in these cells. In comparison with control-treated cells, inhibition of NF- $\kappa$ B activity by BAY 11-7082 during IL-17 treatment for 24-48 h resulted in changes in protein expression characterized by increased expression of E-cadherin and reduced expression of vimentin shown by Western blot analysis (**Figure 3G-I**). These data indicate that inhibition of NF- $\kappa$ B can inhibit IL-17-induced EMT A549 cells.

### *IL-17-induced EMT via ZEB1 depends on NF- $\kappa$ B activation in A549*

The gene expression profile mediating EMT is regulated by one or more transcription factors, including Twist, ZEB1, Slug, and Snail. These factors are transcriptionally induced by upstream signaling pathways, including NF- $\kappa$ B [37]. This prompted us to investigate, through Western blot analysis, the differential expression of these factors in A549 cells during IL-17 treatment compared to untreated controls. A549 cells treated with IL-17 exhibited substantial upregulation of ZEB1 compared to untreated cells (**Figure 2A**). Pharmacologic inhibition of IKK by BAY 11-7082 in A549 cells resulted in a time-dependent decrease in ZEB1 expression but no notable changes in Twist, Slug, or Snail expression. These results indicate that an increase in NF- $\kappa$ B can lead to ZEB1 overexpression in IL-17-treated A549 cells (**Figure 3G, 3H**). These findings revealed that the augmented expression of ZEB1 that occurs in A549 cells during IL-17 treatment is mediated by increased NF- $\kappa$ B activity.

### *IL-17 promotes the migration and invasion of A549*

To further investigate the effects of IL-17 on invasion and migration, we performed a wound healing assay and Transwell invasion assay in A549 cells (IL-17, IL-17 + BAY 11-7028, and IL-17 + ZEB1 shRNA) and control cell. As shown in **Figure 4A**, the wound healing capacity was

significantly increased in A549 cells with IL-17 treatment for 24 h compared to the other three groups (control, IL-17 + BAY 11-7028, and IL-17 + ZEB1 shRNA). Moreover, the Transwell invasion assay revealed that ZEB1 shRNA and NF- $\kappa$ B inhibitor BAY 11-7028 treatments suppressed IL-17-induced cell migration and invasion (**Figure 4A and 4B**,  $P < 0.01$ ). These data further suggest that increased IL-17 levels in lung cancer cells can promote cell migration and invasion via the NF- $\kappa$ B/ZEB1 signaling pathway.

## Discussion

In this study, we provide evidence that IL-17 induces epithelial mesenchymal transition (EMT) in lung cancer cells A549 and that this effect is mediated through ZEB1 activation. EMT is an active focus of current cancer research with a large body of *in vitro*, animal, and patient evidence strongly supporting a key role for EMT in cancer progression and metastasis [38-40].

Cumulative evidence has highlighted a link between IL-17 and EMT in pulmonary fibrogenesis associated with idiopathic pulmonary fibrosis (IPF) [41, 42]. It is a logical hypothesis that EMT may also be involved in IL-17-induced invasion and metastasis in lung cancer. To confirm this hypothesis, a series of *in vitro* assays, including wound healing and Matrigel invasion assays, were used to investigate the role of IL-17 in the regulation of lung cancer cell invasion and metastasis. Interestingly, IL-17 treatment had a significant impact on EMT, as evidenced by increased expression of mesenchymal markers (vimentin) and decreased expression of epithelial markers (E-cadherin). Induction of EMT by IL-17 was further confirmed by morphological analysis of the cells treated with IL-17, which showed changes from characteristic cobblestone-like epithelial morphology to spindle- and fibroblast-like shapes. These observations provided compelling evidence to further support our emerging view that IL-17 levels is an important factor in lung cancer cell aggressiveness, likely by facilitating EMT to promote higher metastatic and invasive abilities.

ZEB1 is a direct transcription repressor of E-cadherin and acts by binding to specific E-boxes of its promoter [32]. ZEB1 has been

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reported to evoke tumorigenic and invasive properties in epithelial cells upon overexpression [33]. Wellner and colleagues have also shown that ectopic expression of ZEB1 results in EMT and enhances motility and invasiveness [31]. In addition, high expression of ZEB1 was frequently found in lung cancer tissues and predicted poor outcome in lung cancer patients. In the present study, A549 lung cancer cells were stimulated by IL-17. The treatment significantly induced the expression of ZEB1, leading to the loss of E-cadherin and the induction of EMT. These results were consistent with the mechanisms of EMT induction. ZEB1 has been termed a master regulator of EMT [18]. A hierarchical model of transcriptional regulation of EMT involves other key transcription factors that have been previously identified, including ZEB1, Snail, Slug, and Twist. Our model places ZEB1 at the top of this hierarchy in lung cancer. We also showed that transfection with small interfering RNA (siRNA) targeting ZEB1 inhibits IL-17-induced vimentin expression in A549 cells.

The transduction pathways involved in EMT include those triggered by NF- $\kappa$ B and many others. As a recent study has indicated a role for IL-17 in activating the NF- $\kappa$ B signaling pathway [29, 30], we sought to determine if this signaling pathway participates in the IL-17-induced EMT process. Our study demonstrated that increased levels of phosphorylation and nuclear translocation of NF- $\kappa$ B is found in A549 cells treated with IL-17. When A549 lung cancer cells were treated with the NF- $\kappa$ B inhibitor BAY 11-7028, the reduction of E-cadherin induced by EMT was greatly attenuated. Thus, our results indicated that the NF- $\kappa$ B signaling pathway is involved in IL-17-induced EMT in A549 cells.

NF- $\kappa$ B activity has been shown to induce ZEB1 overexpression in breast cancer cells [27]. Also, ectopic expression of IL-17 results in activation of the NF- $\kappa$ B signaling pathway in vascular smooth muscle cells [29, 30]. Hence, we examined whether NF- $\kappa$ B is involved in IL-17-induced upregulation of ZEB1 in lung cancer cells. Inhibition of NF- $\kappa$ B by a specific inhibitor led to a significant reduction of ZEB1 expression in IL-17-treated A549 cells, suggesting that IL-17 stimulated expression of ZEB1, at least partly, through activation of the NF- $\kappa$ B signaling pathway in A549 lung cancer cells. Furthermore, the wound healing assay and Transwell invasion

assay was performed in the four groups mentioned above, and IL-17 was further confirmed to promote invasion and migration of these cells. Future research should address if NF- $\kappa$ B directly binds to ZEB1, thereby controlling the transcription of ZEB1 in lung cancer cells.

In summary, the results of this study indicate that IL-17 promotes lung cancer cell migration and invasion through NF- $\kappa$ B-mediated upregulation of ZEB1. Our findings provide new insights on how IL-17 enhances the invasive phenotype of lung cancer cells. On these grounds, we propose that targeting the IL-17/NF- $\kappa$ B/ZEB1 pathway may be a potential therapeutic strategy to prevent lung cancer invasion and metastasis.

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### Disclosure of conflict of interest

None.

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### References

- [1] Marugame T, Yoshimi I. Comparison of cancer mortality (lung cancer) in five countries: France, Italy, Japan, UK and USA from the WHO Mortality Database (1960-2000). *Jpn J Clin Oncol* 2005; 35: 168-170.
- [2] Balkwill F. Cancer and the chemokine network. *Nature Rev Cancer* 2004; 4: 540-550.
- [3] Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. *J Clin Invest* 2007; 117: 1175-1183.
- [4] Levina V, Marrangoni AM, DeMarco R, Gorelik E, Lokshin AE. Drug-selected human lung cancer stem cells: cytokine network, tumorigenic

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- and metastatic properties. *PLoS One* 2008; 3: e3077.
- [5] Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; 420: 860-867.
- [6] He W, Liu Q, Wang L, Chen W, Li N, Cao X. TLR4 signaling promotes immune escape of human lung cancer cells by inducing immunosuppressive cytokines and apoptosis resistance. *Mol Immunol* 2007; 44: 2850-2859.
- [7] Tosolini M, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, Berger A, Bruneval P, Fridman WH, Pagès F. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res* 2011; 71: 1263-1271.
- [8] Zhang B, Rong G, Wei H, Zhang M, Bi J, Ma L, Xue X, Wei G, Liu X, Fang G. The prevalence of Th17 cells in patients with gastric cancer. *Biochem Biophys Res Commun* 2008; 374: 533-537.
- [9] Zhang JP, Yan J, Xu J, Pang XH, Chen MS, Li L, Wu C, Li SP, Zheng L. Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients. *J Hepatol* 2009; 50: 980-989.
- [10] Chen X, Wan J, Liu J, Xie W, Diao X, Xu J, Zhu B, Chen Z. Increased IL-17-producing cells correlate with poor survival and lymphangiogenesis in NSCLC patients. *Lung Cancer* 2010; 69: 348-354.
- [11] Numasaki M, Watanabe M, Suzuki T, Takahashi H, Nakamura A, McAllister F, Hishinuma T, Goto J, Lotze MT, Kolls JK. IL-17 enhances the net angiogenic activity and in vivo growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis. *J Immunol* 2005; 175: 6177-6189.
- [12] Li Q, Han Y, Fei G, Guo Z, Ren T, Liu Z. IL-17 promoted metastasis of non-small-cell lung cancer cells. *Immunol Lett* 2012; 148: 144-150.
- [13] Sabbah M, Emami S, Redeuilh G, Julien S, Prévoist G, Zimmer A, Ouelaa R, Bracke M, De Wever O, Gespach C. Molecular signature and therapeutic perspective of the epithelial-to-mesenchymal transitions in epithelial cancers. *Drug Resist Update* 2008; 11: 123-151.
- [14] Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; 139: 871-890.
- [15] Voulgari A, Pintzas A. Epithelial-mesenchymal transition in cancer metastasis: mechanisms, markers and strategies to overcome drug resistance in the clinic. *Biochim Biophys Acta Rev Cancer* 2009; 1796: 75-90.
- [16] Iwatsuki M, Mimori K, Yokobori T, Ishi H, Beppu T, Nakamori S, Baba H, Mori M. Epithelial-mesenchymal transition in cancer development and its clinical significance. *Cancer Sci* 2010; 101: 293-299.
- [17] Yilmaz M, Christofori G. Mechanisms of motility in metastasizing cells. *Mol Cancer Res* 2010; 8: 629-642.
- [18] Takeyama Y, Sato M, Horio M, Hase T, Yoshida K, Yokoyama T, Nakashima H, Hashimoto N, Sekido Y, Gazdar AF. Knockdown of ZEB1, a master epithelial-to-mesenchymal transition (EMT) gene, suppresses anchorage-independent cell growth of lung cancer cells. *Cancer Lett* 2010; 296: 216-224.
- [19] Gemmill RM, Roche J, Potiron VA, Nasarre P, Mitas M, Coldren CD, Helfrich BA, Garrett-Mayer E, Bunn PA, Drabkin HA. ZEB1-responsive genes in non-small cell lung cancer. *Cancer Lett* 2011; 300: 66-78.
- [20] Hurt EM, Saykally JN, Anose BM, Kalli KR, Sanders MM. Expression of the ZEB1 ( $\delta$ EF1) transcription factor in human: additional insights. *Mol Cell Biochem* 2008; 318: 89-99.
- [21] Kumar M, Allison DF, Baranova NN, Wamsley JJ, Katz AJ, Bekiranov S, Jones DR, Mayo MW. NF-kappaB regulates mesenchymal transition for the induction of non-small cell lung cancer initiating cells. *PLoS One* 2013; 8: e68597.
- [22] Min C, Eddy SF, Sherr DH, Sonenshein GE. NF-kappaB and epithelial to mesenchymal transition of cancer. *J Cell Biochem* 2008; 104: 733-744.
- [23] Julien S, Puig I, Caretti E, Bonaventure J, Nelles L, Van Roy F, Dargemont C, De Herreros AG, Bellacosa A, Larue L. Activation of NF-kB by Akt upregulates Snail expression and induces epithelium mesenchyme transition. *Oncogene* 2007; 26: 7445-7456.
- [24] Maier HJ, Schmidt-Strassburger U, Huber MA, Wiedemann EM, Beug H, Wirth T. NF-kappaB promotes epithelial-mesenchymal transition, migration and invasion of pancreatic carcinoma cells. *Cancer Lett* 2010; 295: 214-228.
- [25] Huber MA, Azoitei N, Baumann B, Grünert S, Sommer A, Pehamberger H, Kraut N, Beug H, Wirth T. NF-kB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. *J Clin Invest* 2004; 114: 569-581.
- [26] Li Q, Liu BC, Lv LL, Ma KL, Zhang XL, Phillips AO. Monocytes induce proximal tubular epithelial-mesenchymal transition through NF-kappa B dependent upregulation of ICAM-1. *J Cell Biochem* 2011; 112: 1585-1592.
- [27] Chua HL, Bhat-Nakshatri P, Clare SE, Morimiya A, Badve S, Nakshatri H. NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. *Oncogene* 2007; 26: 711-724.
- [28] Katoh M. Integrative genomic analyses of ZEB2: Transcriptional regulation of ZEB2

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- based on SMADs, ETS1, HIF1 $\alpha$ , POU/OCT, and NF- $\kappa$ B. *Int J Oncol* 2009; 34: 1737-1742.
- [29] Li G, Zhang Y, Qian Y, Zhang H, Guo S, Sunagawa M, Hisamitsu T, Liu Y. Interleukin-17A promotes rheumatoid arthritis synoviocytes migration and invasion under hypoxia by increasing MMP2 and MMP9 expression through NF- $\kappa$ B/HIF-1 $\alpha$  pathway. *Mol Immunol* 2013; 53: 227-236.
- [30] Cheng G, Wei L, Xiurong W, Xiangzhen L, Shiguang Z, Songbin F. IL-17 stimulates migration of carotid artery vascular smooth muscle cells in an MMP-9 dependent manner via p38 MAPK and ERK1/2-dependent NF- $\kappa$ B and AP-1 activation. *Cell Mol Neurobiol* 2009; 29: 1161-1168.
- [31] Wellner U, Schubert J, Burk UC, Schmalhofer O, Zhu F, Sonntag A, Waldvogel B, Vannier C, Darling D, zur Hausen A. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nature Cell Biol* 2009; 11: 1487-1495.
- [32] Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, Brabletz T. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO Rep* 2008; 9: 582-589.
- [33] Ohashi S, Natsuzaka M, Naganuma S, Kagawa S, Kimura S, Itoh H, Kalman RA, Nakagawa M, Darling DS, Basu D. A NOTCH3-mediated squamous cell differentiation program limits expansion of EMT-competent cells that express the ZEB transcription factors. *Cancer Res* 2011; 71: 6836-6847.
- [34] Sánchez-Tilló E, de Barrios O, Siles L, Cuatrecasas M, Castells A, Postigo A.  $\beta$ -catenin/TCF4 complex induces the epithelial-to-mesenchymal transition (EMT)-activator ZEB1 to regulate tumor invasiveness. *Proc Natl Acad Sci USA* 2011; 108: 19204-19209.
- [35] Corvaisier-Chiron M, Beauvillain C. Les lymphocytes T régulateurs et les lymphocytes Th17: fonctions physiologiques et pathologiques. *Revue Francophone des Laboratoires* 2010; 2010: 31-40.
- [36] Samson M, Lakomy D, Audia S, Bonnotte B. [T(H)17 lymphocytes: induction, phenotype, functions, and implications in human disease and therapy]. *Rev Méd Interne* 2011; 32: 292-301.
- [37] Naugler WE, Karin M. NF- $\kappa$ B and cancer—identifying targets and mechanisms. *Curr Opin Genet Dev* 2008; 18: 19-26.
- [38] Yilmaz M, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. *Cancer Metastasis Rev* 2009; 28: 15-33.
- [39] Sahai E. Mechanisms of cancer cell invasion. *Curr Opin Genet Dev* 2005; 15: 87-96.
- [40] Wolf K, Mazo I, Leung H, Engelke K, von Andrian UH, Deryugina EI, Strongin AY, Bröcker EB, Friedl P. Compensation mechanism in tumor cell migration mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J Cell Biol* 2003; 160: 267-277.
- [41] Mi S, Li Z, Yang HZ, Liu H, Wang JP, Ma YG, Wang XX, Liu HZ, Sun W, Hu ZW. Blocking IL-17A promotes the resolution of pulmonary inflammation and fibrosis via TGF- $\beta$ 1-dependent and -independent mechanisms. *J Immunol* 2011; 187: 3003-3014.
- [42] Vittal R, Fan L, Greenspan DS, Mickler EA, Gopalakrishnan B, Gu H, Benson HL, Zhang C, Burlingham W, Cummings OW, Wilkes DS. IL-17 induces type V collagen overexpression and EMT via TGF- $\beta$ -dependent pathways in obliterative bronchiolitis. *Am J Physiol Lung Cell Mol Physiol* 2013; 304: L401-L414.