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YB-1 overexpression promotes a TGF-β1-induced epithelial–mesenchymal transition via Akt activation

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ABSTRACT

The Y-box binding protein-1 (YB-1) is a transcription/translation regulatory protein, and the expression thereof is associated with cancer aggressiveness. In the present study, we explored the regulatory effects of YB-1 during the transforming growth factor-β1 (TGF-β1)-induced epithelial-to-mesenchymal transition (EMT) in lung adenocarcinoma cells. Downregulation of YB-1 increased E-cadherin promoter activity, and upregulation of YB-1 decreased promoter activity, suggesting that the YB-1 level may be correlated with the EMT. TGF-β1 induced YB-1 expression, and TGF-β1 translocated cytosolic YB-1 into the nucleus. YB-1 overexpression promoted TGF-β1-induced downregulation of epithelial markers, upregulation of mesenchymal markers, and cell migration. Moreover, YB-1 overexpression enhanced the expression of E-cadherin transcriptional repressors via TGF-β1-induced Akt activation. Our findings afford new insights into the role played by YB-1 in the TGF-β1 signaling pathway.

1. Introduction

Transforming growth factor (TGF)-β1, a multipotent cytokine, regulates many cellular functions, including apoptosis, differentiation, and proliferation [1]. TGF-β1 promotes tumor progression, particularly by functioning as a primary inducer of the epithelial–mesenchymal transition (EMT) [2]. The EMT is characterized by morphological changes attributable to loss of epithelial features, acquisition of mesenchymal features, dissolution of cell–cell interactions, increased cell motility, and synthesis of extracellular matrix [3–5]. TGF-β1 induces the EMT by activating several transcription factors, such as Snail, Slug, Twist1, and zinc finger E-box-binding homeobox 1 (ZEB1), via either Smad or non-Smad pathways [6–9]. Such overexpression represses E-cadherin expression [10].

Y-box binding protein-1 (YB-1) is a DNA- and RNA-binding protein involved in regulation of DNA replication and repair, transcription, pre-mRNA splicing, mRNA translation, and mRNA stability [11–13]. YB-1 plays various biological roles in both the nucleus and cytoplasm [14,15]. When in the nucleus, YB-1 exhibits oncogenic functions by regulating the transcription of many genes involved in cell division, the immune response, multidrug resistance, apoptosis, and tumor growth [16,17]. YB-1 binds to the Y-box sequence in the promoter of the multidrug resistance (*MDR1*) gene, which encodes a P-glycoprotein, and stimulates its transcription [18,19]. In addition, YB-1, complexed with activating protein (AP)-2 and p53, binds to the promoter of the *metalloproteinase 2* gene, activating transcription thereof [20,21].

In contrast, cytosolic YB-1 exhibits potent tumor–suppressive activity. YB-1 forms complexes with messenger ribonucleoproteins (mRNPs) and regulates mRNA translation, stability, and localization [12,22]. YB-1 regulates the translation of several mRNAs required for the EMT, including *Snail* mRNA [23]. Moreover, YB-1 binds to both the 5′ and 3′ untranslated regions (UTRs) of vascular endothelial growth factor (*VEGF*) mRNA, together with the polypyrimidine tract binding protein (PTB), stabilizing *VEGF* mRNA [24].

Aggressive and advanced tumors frequently overexpress YB-1 [25,26]. Therefore, YB-1 may serve as a prognostic marker of tumor aggressiveness [18,26]. In breast cancer, YB-1 stimulates tumor metastasis and the EMT [27]. When the Ras signaling pathway is activated, YB-1 overexpression triggers the EMT, featuring the loss of epithelial markers and induction of mesenchymal markers [23]. Although the significance of YB-1 has been addressed in the context of tumorigenesis, including the EMT, little is known about the role played by YB-1 in TGF-β1-mediated EMT. In the present study, we investigated the effect of YB-1 on TGF-β1-induced EMT in A549 lung carcinoma cells.

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2. Materials and methods

2.1. Materials

Cell culture reagents were purchased from WelGENE (Daegu, Korea), Lipofectamine™ 3000 and TRIzol® were from Invitrogen (Carlsbad, CA), and recombinant human TGF-β1 was purchased from PeproTech (Rocky Hill, NJ). Antibodies against E-cadherin and N-cadherin were from BD Biosciences (Franklin Lakes, NJ). Anti-YB-1 was from Abcam (Cambridge, UK). β-actin and Flag antibodies were from Sigma (St. Louis, MO, USA). Alexa Fluor 546-labeled anti-mouse IgG was from Life Technologies (Grand Island, NY). Control and YB-1 small interfering RNAs (siRNAs) were from Genolution Pharmaceuticals (Seoul, Korea). A High-Capacity cDNA Reverse Transcription kit was purchased from Applied Biosystems (Carlsbad, CA), and an SYBR® Green Quantitative PCR kit was from Bio-Rad (Berkeley, CA, USA).

2.2. Cloning, cell culture, and transfection

The human *YB-1* gene was cloned via polymerase chain reaction (PCR) from a human placental cDNA library. The resulting fragment was ligated into pCS4-3Flag and pLNCX2 vectors, and insert integrity verified by sequencing. The human lung carcinoma cell line A549 was grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS) with penicillin (100 units/mL) and streptomycin (100 μg/mL), at 37 °C under 5% CO_2. Transfections of the indicated plasmids proceeded using the Lipofectamine™ reagent according to the manufacturer's instructions. Stable transfected clones were selected with G418 (800 μg/mL). The sequences of the two YB-1 siRNA (siYB-1) oligonucleotides were as follows: siYB-1#1, 5′-UUUGCUGGUAAUUGCGUGGAGGACC-3; ′ siYB-1#2, 5′-UACUGUGGUCGACGCCCAUAGGGUC-3.′

2.3. Luciferase reporter assay

A549 cells in six-well plates were transfected with 500 ng of an E-cadherin luciferase reporter construct. Where indicated, co-transfection featured the pCS4-Flag vector, pCS4-Flag-YB-1, or siRNA. All cells were co-transfected with 200 ng of a β-galactosidase-encoding plasmid to allow transfection efficiency to be calculated. At 48 h after transfection, luciferase activities within cells were determined via luminometry, using a luciferase assay system (Promega, Madison, WI) according to the manufacturer's protocol. The luciferase assay was performed in triplicate in each of three independent experiments.

2.4. Western blot analysis

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 50 mM EDTA, 1% Triton X-100) containing a protease inhibitor cocktail (GenDEPOT, Barker, TX, USA) at 4 °C. After centrifugation at 12,000 rpm for 15 min, supernatants were collected. Protein concentrations were measured via Bradford's method. Equal amounts of proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred onto nitrocellulose membranes. These membranes were blocked with 5% skim milk and incubated with primary antibodies as indicated. After washing, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) system.

2.5. Immunofluorescence staining

A549 cells on glass cover slips were fixed in 4% formaldehyde in PBS for 15 min. The cells were washed three times with PBS; autofluorescence was quenched by addition of 0.1 M glycine in PBS; and the cells were permeabilized with 0.2% Triton X-100 for 20 min. After washing with PBS, cells were blocked by incubation with 5% bovine serum albumin (BSA) in PBS for 1 h, and next incubated overnight with an anti-YB-1 antibody, at 4 °C. The cells were washed three times with PBS, incubated with Alexa Fluor 546-labeled anti-mouse IgG (the secondary antibody), and the nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI). The cells were then observed under a confocal fluorescence microscope.

2.6. Wound healing assays

A549 cells grown in six-well plates and cells stably expressing pLNCX2 or the Flag-YB-1 vector were scratched and incubated with TGF-β1 for the indicated times. Cell morphologies were observed under inverted phase-contrast microscopy. Three random fields in each well were photographed, and migrated cells counted.

2.7. Quantitative real-time PCR

Total RNAs were extracted from transfected cells using TRIzol® and treated with DNase I. First-strand cDNA was synthesized from 1 μg of total RNA using a High-Capacity cDNA Reverse Transcription kit. Snail and Slug mRNA levels were quantified by real-time PCR using a SYBR® Green Quantitative PCR kit (Bio-Rad) with the aid of a C1000™ thermal cycler, following the manufacturer's protocol. The PCR primer sequences were as follows: human Snail, forward 5′-CCCCAATCGGAAGCCTAACT-3′ and reverse 5′-CGTAGGGCTGCTGGAAGGTA-3; ′ and human Slug, forward 5′-CCATTCCACGCCCAGCTA-3′ and reverse 5′-CTCACTCGCCCCAAAGATGA-3.′ Each sample was tested in triplicate, and the human cyclophilin gene served as a normalization control.

3. Results and discussion

3.1. YB-1 regulates E-cadherin expression in A549 cells

To investigate the role played by YB-1 during the EMT, we explored the effect of YB-1 on the expression levels of EMT marker proteins, E-cadherin (an epithelial marker) and N-cadherin (a mesenchymal marker). After A549 cells were transfected with control or YB-1 siRNA, we measured E-cadherin promoter activity using the luciferase assay. As shown in Fig. 1A, such promoter activity was increased in YB-1 knockdown cells compared to cells transfected with control siRNA. Conversely, YB-1 overexpression significantly decreased E-cadherin promoter activity (Fig. 1B). In addition, to verify the effect of YB-1 on the EMT, we overexpressed YB-1 in A549 cells via stable transfection. Interestingly, YB-1 overexpression decreased E-cadherin protein levels and increased N-cadherin levels (Fig. 1C). Thus, the YB-1 level and the EMT were correlated.

3.2. TGF-β1 induces upregulation and nuclear localization of YB-1 in A549 cells

To determine whether the observed changes in YB-1 expression and localization were associated with the TGF-β1-mediated EMT, we explored the effect of TGF-β1 on the expression levels and localization of YB-1 in A549 cells. Such cells were treated with 5 ng/mL TGF-β1 for 0, 3, 6, 12, 24, or 48 h, and YB-1 protein levels measured by Western blotting. As shown in Fig. 2A, YB-1 was upregulated in response to TGF-β1, in a time-dependent manner.

To determine YB-1 localization in response to TGF-β1, A549 cells were treated with TGF-β1 for 12 h and the YB-1 location determined via immunofluorescence. In untreated cells, YB-1 was predominantly cytoplasmic. However, TGF-β1 increased nuclear YB-1 levels (Fig. 2B). In the absence of TGF-β1, ~95% of cellular YB-1 was in the cytoplasm; upon TGF-β1 treatment, we observed a ~65% increase in nuclear staining. Thus, a TGF-β1-induced effect on YB1 may be involved in control of the TGF-β1-induced EMT.

3.3. YB-1 overexpression triggers a TGF-β1-induced EMT and cell migration

To explore whether YB-1 was involved in the TGF-β1-induced EMT, we measured the effects of exogenous YB-1 overexpression in A549 cells. Upon TGF-β1 addition, YB-1 overexpression accelerated E-cadherin downregulation and N-cadherin upregulation, compared to control vector-transfected cells (Fig. 3A). To confirm the role played by YB-1 in the EMT, we performed similar experiments in stably transfected cells. As shown in Fig. 3B, the TGF-β1-induced E-cadherin downregulation and N-cadherin upregulation were enhanced in YB-1-overexpressing A549 cells. To further characterize this phenomenon, we performed a wound healing assay. After TGF-β1 treatment, YB-1 overexpression triggered high-level cell migratory activity, and earlier wound closure, in a time-dependent manner (Fig. 3C and D). Thus, YB-1 is required for the TGF-β1-mediated EMT and cell migration in A549 cells.

3.4. YB-1 regulates Snail and Slug expression via Akt activation

TGF-β1 activates many EMT-promoting transcription factors, including Snail, Twist, and ZEB, via either Smad or non-Smad pathways [6,28]. Such transcription factors repress E-cadherin expression during the EMT [10]. Activation of non-Smad signaling

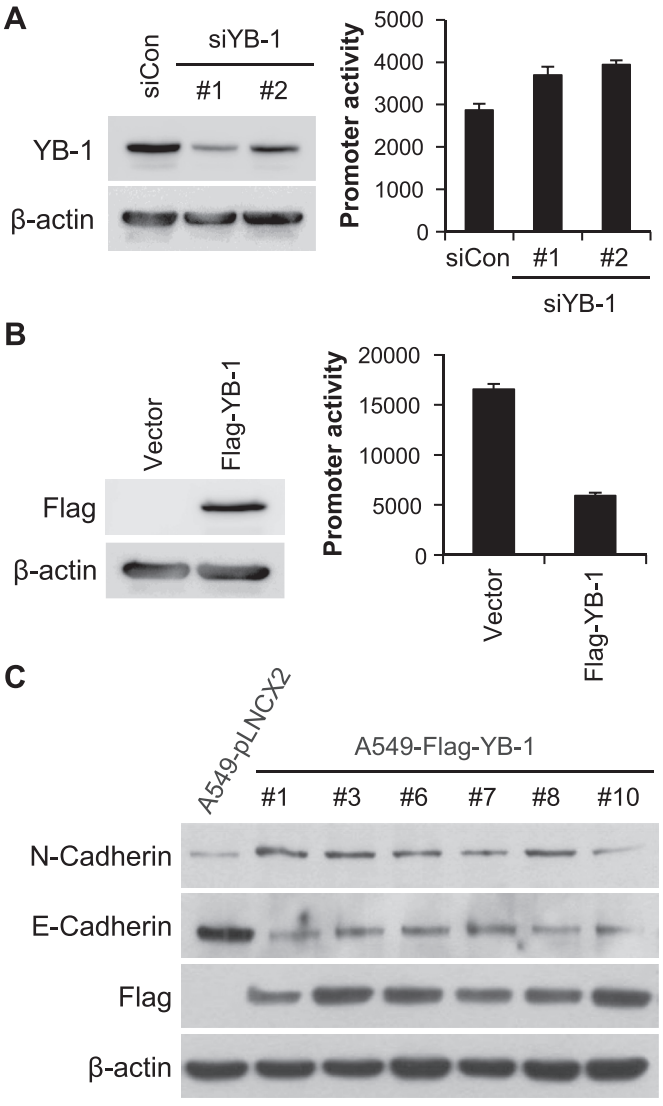

Fig. 1. YB-1 protein level regulates E-cadherin levels in A549 cells. (A) A549 cells were co-transfected with pE-cadherin-Luc and control siRNA (siCon) or YB-1 siRNA (siYB-1#1 and siYB-1#2). Western blotting quantitated YB-1 expression levels (left) and luciferase activity was also measured (right). (B) After co-transfection with the pCS4-Flag vector (Vector), or pCS4-Flag-YB-1 (Flag-YB-1) carrying pE-cadherin-Luc, Flag-YB-1 protein expression was examined via Western blotting (left), and E-cadherin promoter activity was measured by assay of luciferase activity (right). The luciferase activity data in (A) and (B) represents the means ± standard deviations (SDs) of data from three independent experiments. (C) YB-1 was overexpressed in A549 cells via stable transfection (retroviral infection). Both N-cadherin and E-cadherin expression were evident in stable A549 clones expressing the pLNCX2 vector or Flag-YB-1.

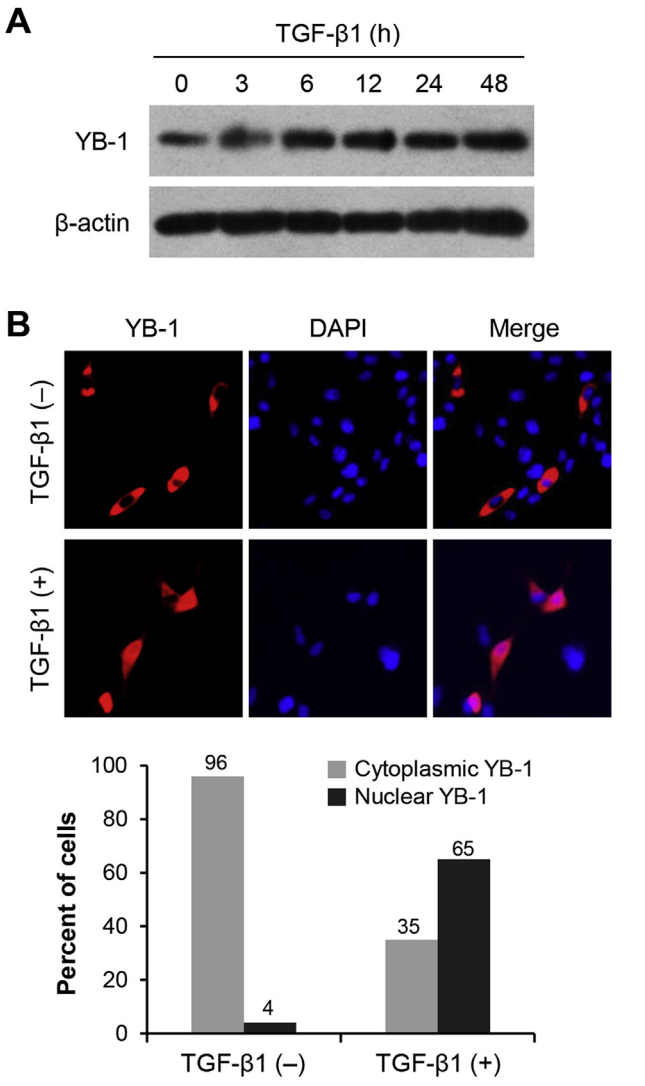

Fig. 2. TGF-β1-induced YB-1 protein levels and nuclear localization. (A) YB-1 protein levels were measured in A549 cells treated with TGF-β1 for the indicated times. β-actin served as a loading control. (B) A549 cells were treated with TGF-β1 (or not) for 12 h, and YB-1 localization was analyzed by immunofluorescence staining. Nuclei were stained with DAPI. The numbers of cells/100 cells exhibiting cytoplasmic or nuclear fluorescence are shown.

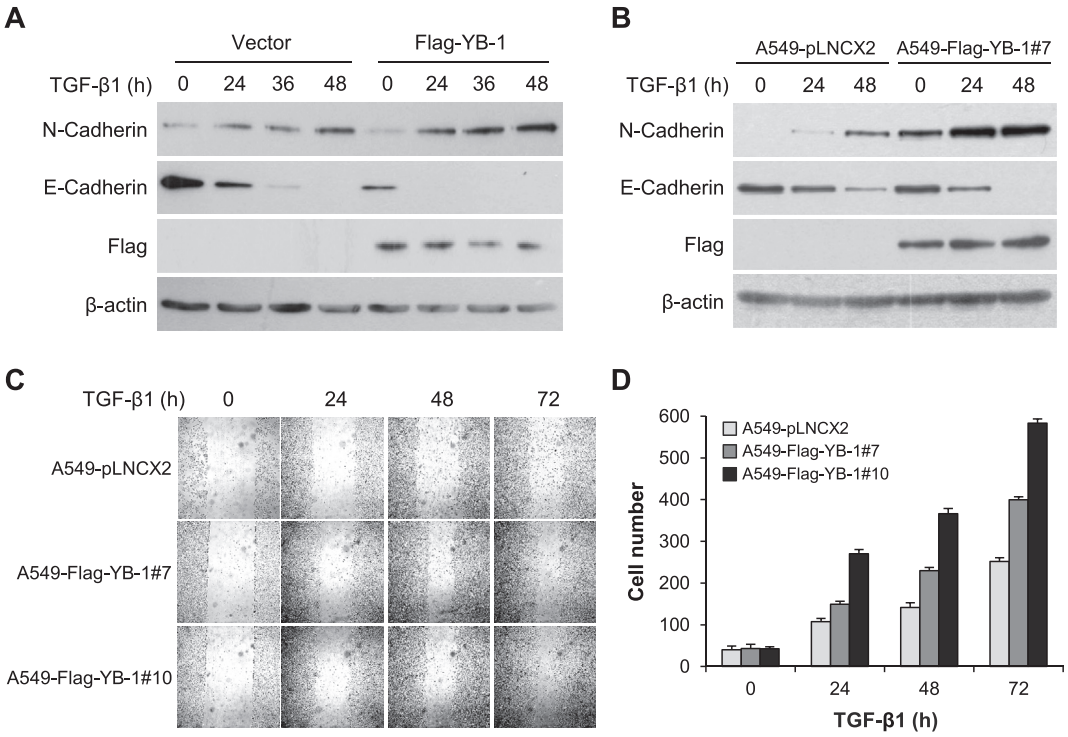

Fig. 3. YB-1 overexpression enhances the TGF-β1-induced EMT and cell migration. (A) Cells transfected with the pCS4-Flag vector or pCS4-Flag-YB-1 were treated with TGF-β1 for the indicated times. The E-cadherin and N-cadherin expression levels were analyzed via Western blotting. β-actin served as a loading control. (B) A549 cells stably expressing the empty vector (A549-pLNCX2) or Flag-YB-1 (A549-Flag-YB-1#7) were treated with TGF-β1 for the indicated times. The E-cadherin and N-cadherin expression levels were analyzed via Western blotting. (C) A549-pLNCX2, A549-Flag-YB-1#7, or A549-Flag-YB-1#10 cells were scratched and incubated with TGF-β1 for the indicated times. (D) The numbers of migrated cells are shown. Cells in three random fields were counted and the numbers summed.

responses, extracellular signal-regulated kinases (ERKs), the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and Rho GTPases, modulate the transcription of both EMT regulators and factors of Smad-dependent pathways [9,29,30]. Thus, we explored whether YB-1 was required for TGF-β1-induced Snail and Slug expression. Quantitative real-time PCR revealed that YB-1 overexpression promoted TGF-β1-induced Snail and Slug expression (Fig. 4A and B), indicating that YB-1 regulated E-cadherin expression via Snail and Slug. We next determined whether YB-1 acted upstream to activate Smad and Akt during the TGF-β1-induced EMT. Western

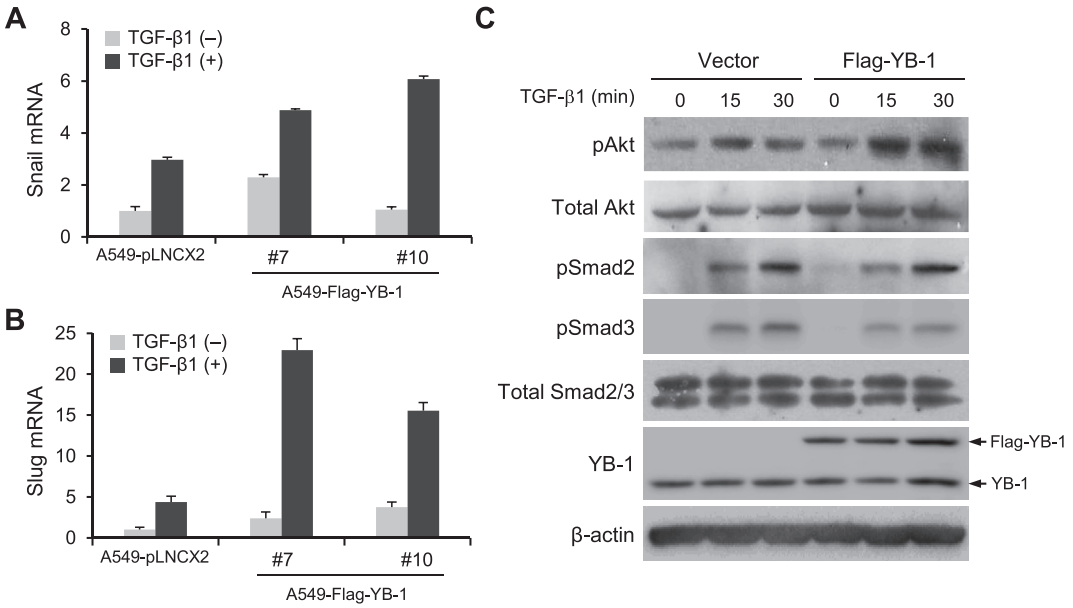

Fig. 4. YB-1 overexpression induces TGF-β1-mediated Snail and Slug expression via Akt activation. (A and B) A549-pLNCX2 and A549-Flag-YB-1 (#7 and #10) cells were treated with TGF-β1 (or not) for 24 h. Quantitative RT-PCR was used to detect Snail and Slug mRNAs. The human cyclophilin gene was used for normalization. The results are shown as -fold changes compared to the levels in A549-pLNCX2 cells. Representative results from three independent experiments are shown. (C) Cells transfected with empty vector or Flag-YB-1 were treated with TGF-β1 for the indicated times. Western blotting was performed to detect phosphorylation of Akt (pAkt) and the Smads (pSmad2 and pSmad3). β-actin served as a loading control.

blotting showed that TGF-β1-induced phosphorylation of Akt was enhanced in YB-1-overexpressing A549 cells, but TGF-β1-induced Smad2/3 phosphorylation was not activated upon YB-1 overexpression (Fig. 4C). These results suggested that YB-1 induced upregulation of Snail and Slug via non-Smad pathways during the TGF-β1-mediated EMT. Therefore, TGF-β1-induced YB-1 expression and nuclear localization upregulates EMT-promoting transcription factors, and furthers the EMT process, in human A549 lung adenocarcinoma cells.

3.5. Summary

We have shown that TGF-β1 increased YB-1 expression and caused cytosolic YB-1 to be translocated into the nucleus. Furthermore, YB-1 overexpression induced a TGF-β1-mediated EMT and cell migration. The YB-1-induced EMT featured upregulation of the Snail and Slug transcription factors, via Akt activation. Our findings suggest that YB-1 may regulate the TGF-β1 signaling pathway.

Conflict of interest

There are no conflict of interest for all authors.

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