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Fyn mediates transforming growth factor-beta1-induced down-regulation of E-cadherin in human A549 lung cancer cells

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ABSTRACT

Transforming growth factor-beta (TGF-β) signaling positively contributes to the regulation of tumor metastasis. However, the underlying molecular mechanisms are less well defined. We here show that Fyn, a member of Src family tyrosine kinases, plays a critical role in mediating TGF-β1-induced down-regulation of E-cadherin in human A549 lung cancer cells. Blockade of Fyn with siRNA knockdown or ligand-binding defective mutant significantly lowered the ability of TGF-β1 to repress E-cadherin expression. Furthermore, our results demonstrated that Fyn facilitates TGF-β1-mediated suppression of E-cadherin through p38 kinase-dependent induction of Snail. Collectively, our findings identify a Fyn-p38-Snail cascade as a new signaling pathway mediating oncogenic TGF-β function.

1. Introduction

The epithelial–mesenchymal transition (EMT), an important morphological event where polarized epithelial cells convert to contractile and motile mesenchymal cells, is recognized as an important process during embryonic development and tissue organization [1,2]. EMT is also known to be an important step in the invasion and metastasis of cancer [3]. Because EMT of cells is associated with loss of cell–cell interaction and formation of tight cell–cell adhesion is mainly dependent on E-cadherin system [4], and loss of E-cadherin is essential for the EMT. Recent studies on the molecular mechanisms by which expression of E-cadherin is down-regulated in epithelial cells reveal that several E box transcriptional repressors, including Snail, Slug, ZEB1, SIP1 and Twist, are involved in this process [5,6]. However, the molecular mechanism by which the expression of these transcription factors is regulated at the beginning of EMT is uncertain.

Transforming growth factor-beta1 (TGF-β1) is a multifunctional cytokine that regulates a wide range of cellular functions, including tissue morphogenesis, differentiation, and extracellular matrix remodeling [7,8]. Recent studies reveal that TGF-β1 functions as a pro-oncogenic factor through induction of EMT [9,10] and TGF-β1-induced EMT in a variety of cells is mediated through Smad or non-Smad signaling pathway. For example, *in vivo* or *in vitro* experiment using either Smad3 knock-out mice or overexpression of a TGF-β receptor type I (TβRI) mutant defective in Smad activation demonstrates a requirement for Smad signaling in mediating EMT [11,12]. In the other hands, the non-Smad signaling pathways, including Ras, RhoA, MAPK, PI3 kinase, Notch, and Wnt, also are implicated in TGF-β1-induced EMT [13–16].

Src family kinases (SFKs) are non-receptor cytoplasmic tyrosine kinases including Src, Yes, Fyn, Blk, Yrk, Fgr, Hck, and Lck and play crucial roles in the regulation of proliferation, aggregation, differentiation, adhesion, motility, and survival of cells [17]. Several recent studies demonstrated that Src, a prototypic member of SFKs, plays a prominent role in invasion and in other tumor progression events such as EMT and development of metastasis [18,19] and, in addition, its increased activity are frequently founded in many types of cancer [20]. In this study, we provide the first evidence that Fyn, one member of SFKs, is required for TGF-β1-mediated down-regulation of E-cadherin in A549 lung cancer cells. Furthermore, our results show that Snail up-regulation via the Fyn-p38 kinase cascade mediates the oncogenic TGF-β signal to down-regulation of E-cadherin.

2. Materials and methods

2.1. Materials

Recombinant TGF-β1was purchased from R&D systems (Minneapolis, MA). The following materials were obtained from Calbiochem (La Jolla, CA): U0126, a MAPK kinase inhibitor, SB203580, a p38 kinase inhibitor, SP600125, a JNK1/2 inhibitor. Small interfering RNAs for control and human Fyn were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

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2.2. Cell culture

A549, a human lung cancer cell line, was obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI1640 medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3. Plasmids, DNA transfection and luciferase assay

Expression vectors encoding pcDNA3-MEK1AA, a dominant negative form of MEK1, and pcDNA3-SEK1KR, a kinase-deficient form of SEK1, were kindly provided by Dr. Gutkind J.S. (National Institutes of Health, Rockville). Recombinant adenoviruses expressing GFP and an inactive form of p38α, p38α (AF), were obtained from Dr. Jiahuai Han (The Scripps Research Institute, California). The E-cadherin promoter-luciferase plasmid DNA, pEcadherin-Luc, was gift from Dr. Kyung Lib Jang (Pusan National University, Korea). The Snail promoter-Luciferase, pSnail-Luc, was kindly provided from Dr. Guhung Jung (Seoul National University, Korea). Expression vectors encoding pAlterMAX-Fyn WT and pAlterMAX-Fyn (P134V/R176K) were gift from Dr. Hamid Band (Harvad Medical School, Boston). The A549 cells were transfected using Polyethyeleimine (Roche, Mannheim, Germany). To control variations in cell numbers and transfection efficiency, all clones were cotransfected with 0.2 μg of CMV-β-GAL, a eukaryotic expression vector in which *Escherichia coli* β-galactosidase (Lac Z) structural gene is under the transcriptional control of the CMV promoter. Luciferase reporter activity was assessed on a luminometer with a luciferase assay system (Promega, Madison, WI) according to the manufacturer's protocol. Transfection experiments were performed in duplicate with two independently isolated sets, and these results were averaged.

2.4. Establishment of stable cell lines

The 19-nucleotide gene-specific sequence spanning from nucleotides 75–93 downstream of the gene transcription start site was selected to suppress human Fyn gene expression. The selected sequence was inserted into a BglII/HindIII-cut pSUPER vector to generate the pSUPER-shFyn vector. Generation of A549-pSUPER and A549-pSUPER-shFyn stable cell lines were performed as previously described [21]. The depletion of endogenous Fyn by shRNA was verified by immunoblot analysis.

2.5. Immunoblot analysis

Immunoblot analysis was done previously [21] using anti-Fyn (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-p38 (Cell Signaling Technology, Beverly, MA), anti-E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Snail (54; Transduction Laboratories, Lexington, KY), anti-FLAG (M5; Sigma Chemical Co., St. Louis, MI), and anti-β-actin (AC-15; Sigma, St. Louis, MO) antibodies.

2.6. Statistical analysis

All data presented are expressed as means ± SD, and a representative of three or more independent experiments. Statistical analyses were by Student's *t*-test for paired data. Results were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Fyn mediates TGF-β1-induced down-regulation of E-cadherin in A549 lung cancer cells

To determine whether Fyn is required for the TGF-β1-induced down-regulation of E-cadherin, we stably silenced the expression of the Fyn gene in A549 non-small lung cancer cells using pSUPER system. As shown in Fig. 1A, siRNA expression mediated by pSUPER vector caused efficient, stable, and specific downregulation of Fyn gene expression in pSUPER-Fyn cells, compared with the pSUPER-Con cells. Upon treatment with TGF-β1, pSUPER-Fyn cells showed a significant rescue in decreased E-cadherin level, compared with the pSUPER-control cells (Fig. 1A). Co-transfection of A549 cells with wild type of Fyn enhanced the TGF-β1-induced suppression of E-cadhrin promoter activity, whereas co-transfection of the cells with Fyn SH2/SH3 double mutant with defective ligand binding capability significantly rescued the decreased activity of the E-cadherin promoter induced by TGF-β1 (Fig. 1B), indicating that Fyn is involved in TGF-β signaling to E-cadherin down-regulation.

3.2. p38 kinase is required for TGF-β1-induced suppression of E-cadherin

TGF-β1-activated TβRI transmit signal through the activation of downstream Smad and non-Smad mitogen-activating protein kinase (MAPK) pathways. Because the involvement of Smad pathway in TGF-β1-induced EMT has been well demonstrated [11,12], we examined if MAPK pathways are responsible for TGF-β1-induced EMT. In order to investigate the role of MAPK pathways in the TGF-β1-induced suppression of E-cadhrin, we employed specific

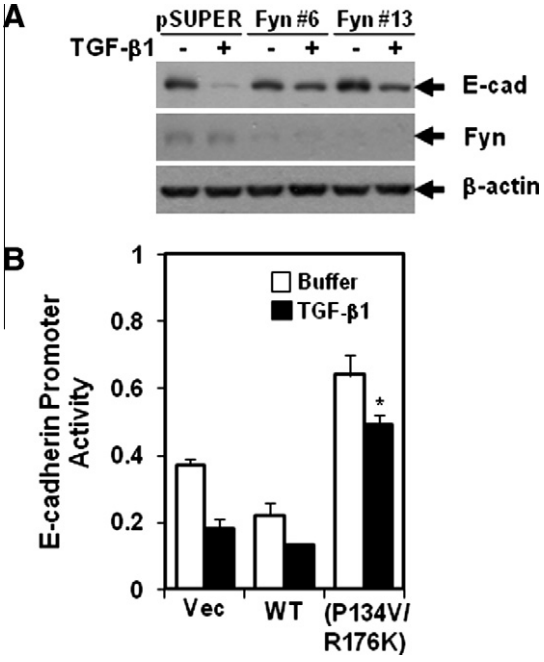

Fig. 1. Fyn is involved in 'the down-regulation of E-cadherin by TGF-β1 in A549 lung cancer cells. (A) A549pSUPER, A549pSUPER-shFyn#6 and A549pSUPER-shFyn#13 cells were stimulated with TGF-β1 (5 ng/ml) for 24 h. Immunoblots of whole cell lysates were probed with E-cadherin, Fyn and β-actin antibodies. (B) Cells were cotransfected with pE-cadherin-Luc and pAlterMAX, pAlterMAX-Fyn WT, or pAlterMAX-Fyn (P134 V/R176 K). At 24 h after transfection, cells were incubated with TGF-β1 (5 ng/ml) for 24 h. Bars depict the mean ± SD of three independent experiments. $^{*}p < 0.05$ Compared with pAlterMAX-transfected cells treated with TGF-β1.

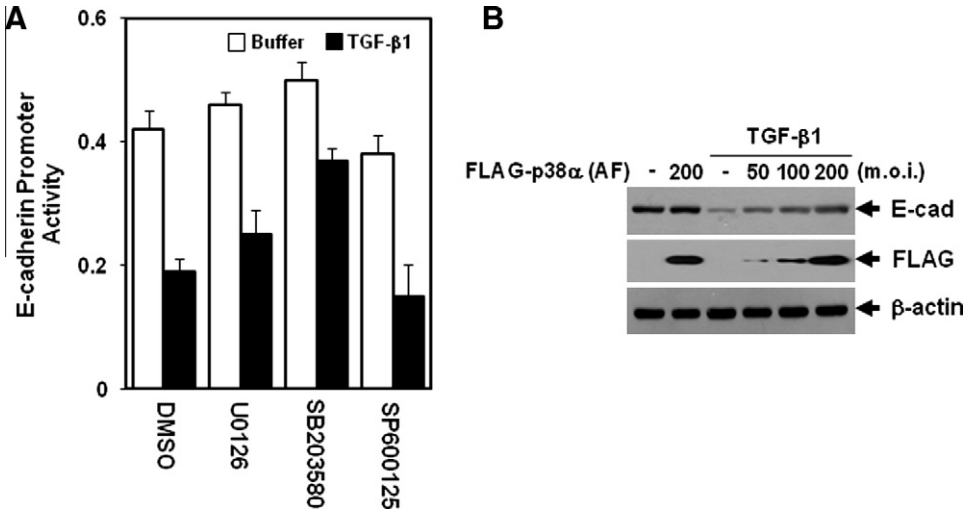

Fig. 2. p38 Kinase is required for repression of TGF-β1-induced E-cadherin expression in A549 cells. (A) Cells were transiently transfected with pE-cadherin Luc reporter plasmid. At 24 h after transfection, cells were incubated with U0126 (10 μM), SB203580 (5 μM), or SP600125 (15 μM) for 12 h before harvest. Luciferase activities were normalized on the basis of β-galactosidase expression to adjust for variation in transfection efficiency. (B) Cells were infected with adenovirus carrying an inactive form of p38α cDNA at indicated concentrations of m.o.i. for 6 h. Then the cells were treated with TGF-β1 (5 ng/ml) for 24 h. Immunoblots of whole cell lysates were probed with E-cadherin, FLAG and β-actin antibodies.

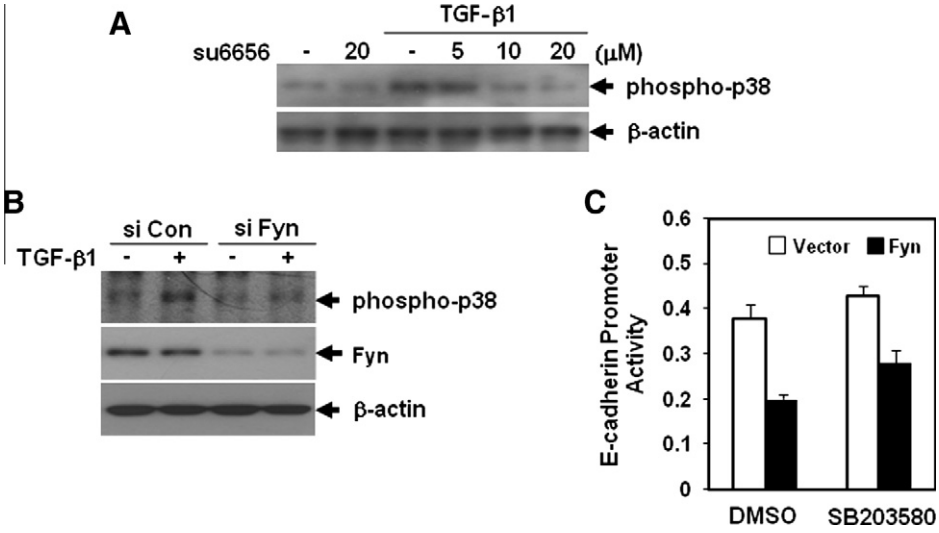

Fig. 3. Fyn acts as an upstream factor for p38 in TGF-β signaling to E-cadherin repression in A549 cells. (A) Cells were pre-treated with su6656 at the indicated concentrations for 30 min, and then incubated with TGF-β1 (5 ng/ml) for 30 min. (B) Subconfluent cells transfected with scramble control siRNA (siCon) or Fyn siRNA (silFyn) were incubated without or with TGF-β1 (5 ng/ml) for 30 min. Immunoblots of whole cell lysates were probed with phosphor-p38, Fyn and β-actin antibodies. (C) Subconfluent cells co-transfected with pE-cadherin-Luc and pAlterMAX or pAlterMAX-Fyn WT were incubated without or with SB203580 (5 μM) for 12 h before harvest. Luciferase activities were normalized on the basis of β-galactosidase expression to adjust for variation in transfection efficiency.

inhibitors for each of these pathways together with TGF-β1. As shown in Fig. 2A, treatment of SB203580, a specific inhibitor of p38, significantly reversed the inhibition of E-cadherin promoter activity by TGF-β1. In contrast, neither U0126, a specific inhibitor of MEK1, nor SP600125, a specific inhibitor of JNK1/2, had a significant effect. Consistently, infection of cells with Adp38α (AF) resulted in reversal changes in expression of E-cadherin upon TGF-β1 treatment (Fig. 2B), suggesting an essential role of p38 on repression of TGF-β1-induced E-cadherin expression.

3.3. Fyn acts upstream of p38 in TGF-β1 signaling to down-regulation of E-cadherin

We next determined whether Fyn acts as an upstream regulator of p38 activation during TGF-β1-induced suppression of E-cadherin expression. Western blot analysis showed that the TGF-β1-induced phosphorylation of p38 was abolished in A549 cells pre-treated with su6656, a specific inhibitor of Fyn, in a dose-dependent manner (Fig. 3A). Consistent with this, TGF-β1-induced phosphorylation of p38 was also blocked in siRNA Fyn-transfected cells, compared with the siRNA Con-transfected cells (Fig. 3B). Furthermore, treatment of cells with SB203580 markedly reversed the inhibitory effect of Fyn on E-cadherin promoter activity (Fig. 3C), indicating an existence of Fyn-p38-linked cascade in TGF-β signaling to down-regulation of E-cadherin gene.

3.4. Snail is involved in the repression of TGF-β1-mediated E-cadherin expression as a downstream target of Fyn-p38-linked signaling

Snail repressor has been shown to be induced by TGF-β1 and to play pivotal roles in E-cadherin repression [22]. We therefore determined whether Fyn is required for the TGF-β1-induced Snail

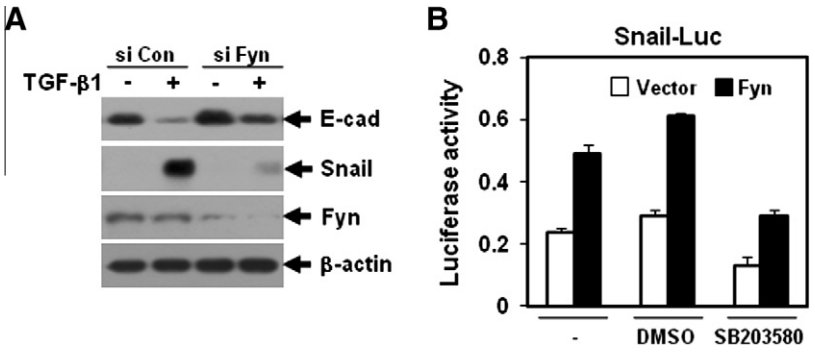

Fig. 4. Snail is a downstream target of Fyn-p38-linked signaling and involved in the TGF-β1-induced repression of E-cadherin. (A) Subconfluent cells transfected with scramble control siRNA (siCon) or Fyn siRNA (silFyn) were incubated without or with TGF-β1 (5 ng/ml) for 24 h. Immunoblots of whole cell lysates were probed with E-cadherin, Snail, Fyn and β-actin antibodies. (B) Subconfluent cells co-transfected with pSnail-Luc and pAlterMAX or pAlterMAX-Fyn WT were incubated without or with SB203580 (5 μM) for 12 h before harvest. Luciferase activities were normalized on the basis of β-galactosidase expression to adjust for variation in transfection efficiency.

expression. As shown in Fig. 4A, induction of TGF-β1-induced Snail expression was substantially reduced by transfection of small interfering RNA (siRNA) specific for Fyn, but not by scrambled siRNA. In addition, the Snail promoter activity was enhanced by cotransfection with Fyn and this effect was strongly inhibited by cotransfection with dominant-negative p38 (Fig. 4B), indicating that induction of Snail by TβRI/Fyn-activated p38 MAPK may be important for TGF-β1-induced repression of E-cadherin expression in A549 human lung cancer cells.

Epithelial–mesenchymal transition (EMT) is a crucial morphological event contributing to the progression of epithelial tumors. It is commonly accepted that TGF-β1 plays an important role in the progression of EMT in some tumor cells. In the present study, we investigated the molecular mechanism whereby Fyn, a non-receptor tyrosine kinase of Src family, mediates the repression of TGF-β1-induced E-cadherin expression in human A549 non-small lung cancer cells. We provide evidences that Fyn mediates TGF-β1-induced suppression of E-cadherin through a signaling pathway dependent on p38 kinase and subsequent Snail up-regulation. Therefore, modulation of Fyn expression may be important therapeutic strategies for the treatment of EMT-related human diseases such as cancer.

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