ARTICLE
Journal of Cellular Biochemistry 113:1501–1513 (2012)

Journal of Cellular Biochemistry

Activation of Notch-1 Enhances Epithelial–Mesenchymal Transition in Gefitinib-Acquired Resistant Lung Cancer Cells

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ABSTRACT

Despite an initial response to EGFR tyrosine kinase inhibitors (EGFR-TKI) in EGFR mutant lung cancer, most patients eventually become resistant and result in treatment failure. Recent studies have shown that epithelial to mesenchymal transition (EMT) is associated with drug resistance and cancer cell metastasis. Strong multiple gene signature data indicate that EMT acts as a determinant of insensitivity to EGFR-TKI. However, the exact mechanism for the acquisition of the EMT phenotype in EGFR-TKI resistant lung cancer cells remains unclear. In the present study, we showed that the expression of Notch-1 was highly upregulated in gefitinib-resistant PC9/AB2 lung cancer cells. Notch-1 receptor intracellular domain (N1IC), the activated form of the Notch-1 receptor, promoted the EMT phenotype in PC9 cells. Silencing of Notch-1 using siRNA reversed the EMT phenotype and restored sensitivity to gefitinib in PC9/AB2 cells. Moreover, Notch-1 reduction was also involved in inhibition of anoikis as well as colony-formation activity of PC9/AB2 cells. Taken together, these results provide strong molecular evidence that gefitinib-acquired resistance in lung cancer cells undergoing EMT occurs through activation of Notch-1 signaling. Thus, inhibition of Notch-1 can be a novel strategy for the reversal of the EMT phenotype thereby potentially increasing therapeutic drug sensitivity to lung cancer cells. J. Cell. Biochem. 113: 1501–1513, 2012.

KEY WORDS: LUNG CANCER; NOTCH-1; EPITHELIAL–MESENCHYMAL TRANSITION; GEFITINIB

Lung cancer is one of the most common causes of death from cancer worldwide. Non-small cell lung cancer (NSCLC), accounting for approximately 80% of all lung cancers, has a poor 5-year survival (15%) due to the aggressiveness of this type of cancer [Cataldo et al., 2011]. Recently, selection of patients using genetic markers and molecular-targeted therapy have shown promising outcomes [Nevins, 2011]. Somatically sensitive mutations in the epidermal growth factor receptor (EGFR) gene in lung adenocarcinoma were associated with a dramatic response to EGFR tyrosine kinase inhibitor (TKI) [Paez et al., 2004]. However, despite

Conflict of interest: No potential conflicts of interest are disclosed.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Science and Technology Planning Project of Guangdong Province, China; Grant number: 83050; Grant sponsor: Medical Scientific Research Foundation of Guangdong Province, China; Grant number: A2009265; Grant sponsor: Cancer Target Therapy Research Fund of Wu Jieping Medial Foundation 2010; Grant number: 320.6720.10015; Grant sponsor: Open Funds of State Key Laboratory of Oncology in Southern China 2010; Grant number: HN2011-07.

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Received 29 June 2011; Accepted 29 November 2011 • DOI 10.1002/jcb.24019 • © 2011 Wiley Periodicals, Inc.
Published online 15 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

an initial response to the treatment in some lung cancer patients with EGFR-TKI, most patients eventually become resistant to the agents, leading to treatment failure. The primary mechanism associated with acquired resistance might be ascribed to a T790M mutation in exon 20 of EGFR [Nevins, 2011]. In addition, MET oncogene amplification and overexpression of hepatic growth factor (HGF) also play roles in drug-acquired resistance [Onitsuka et al., 2010]. One in vitro study showed that the mesenchymal phenotype is more resistant to EGF-TKI than the epithelial phenotype [Uramoto et al., 2010].

Epithelial to mesenchymal transition (EMT) has been identified as a unique process whereby epithelial cells undergo remarkable morphologic changes characterized by a transition from an epithelial cobblestone phenotype to elongated fibroblastic phenotype (mesenchymal phenotype), leading to increased motility and invasion [Thiery et al., 2009]. EMT involves a loss of the epithelial cell–cell junction, reorganization of the actin cytoskeleton, and up-regulation of mesenchymal molecular markers such as fibronectin, α-smooth muscle actin (SMA), Vimentin, and N-cadherin [Denlinger et al., 2010]. Disassembly of cell–cell junctions, including down-regulation and relocation of E-cadherin and β-catenin from cell membrane to the nucleus results in EMT [Yilmaz and Christofori, 2009]. A number of factors that transcriptionally repress E-cadherin have emerged as potent EMT stimulators during normal and cancer development. These include the zinc finger Snail homologues (Snail1, Snail2/Slug, and Snail3) and several basic helix–loop–helix factors including Twist, ZEB1, ZEB2/SIP1, and TCF3/E47/E12 [Casas et al., 2011]. EMT is a dynamic process triggered by the interplay of extracellular signals (such as collagen). Many signaling pathways, including the Wnt pathway, transforming growth factor-β (TGF-β), Hedgehog, and nuclear factor-kappa B (NF-κB) are critical for EMT induction [Luna-Zurita et al., 2010]. However, the exact mechanism for the acquisition of the EMT phenotype of EGFR-TKI resistant lung cancer cells remains unknown.

In this study, we investigated whether the Notch-1 signaling pathway is involved in the acquisition of the EMT phenotype of gefitinib-acquired resistant lung cancer cells. Notch-1 inhibition is involved in decreased invasive behavior and partial reversal of the EMT phenotype in lung adenocarcinoma cells, which was associated with increased expression of E-cadherin, while decreasing expression of Vimentin and Snail. These results suggest that activation of Notch-1 signaling is critical in gefitinib-acquired resistance and the EMT phenotype in lung adenocarcinoma cells.

MATERIALS AND METHODS

CELL LINES AND REAGENTS

The PC9 cell line (harboring EGFR exon 19 deletion) was obtained from the American Type Culture Collection (Manassas, VA). PC9/AB2 cells (gefitinib-acquired resistant PC9 cells) were generously provided by Dr. Caicun Zhou (Shanghai Pulmonary Hospital, Tongji University, China) [Ju et al., 2010]. The gefitinib-resistant NSCLC subline PC9/AB2 was derived from PC9 cells according to the method in another study [Koizumi et al., 2005] and cultured with 2 mmol/L of gefitinib for an additional 6 months. Resistance of PC9/AB2 cells to gefitinib was maintained for at least one year in the medium without gefitinib. All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) and 2 mmol/L L-glutamine. DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester, Sigma–Aldrich Co.), a benzodiazepine-type compound that acts as a selective and non-competitive inhibitor of γ secretase. Gefitinib, (*N*-(3-chloro-4-fluoro-phenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy) quinazolin-4-amine, Tocris Bioscience), is a selective inhibitor of EGFR tyrosine kinase.

CYTOTOXICITY AND CELL PROLIFERATION ASSAYS

For cytotoxicity assays, cell viability was assessed using a tetrazolium salt (WST-8)-based colorimetric assay in the Cell Counting Kit 8 (CCK-8; Dojindo Molecular Technology, Japan). Briefly, cells were seeded onto 96-well plates at an initial density of 5×10^3 cells/well for 24 h and cultured with increased concentrations of gefitinib for an additional 48 h. For cell proliferation assays, PC9 cells and PC9/AB2 cells (1×10^4/well, respectively) were seeded onto six-well plates for 5–7 days. Afterwards, 10 μl of CCK-8 solution was then added and incubated for 1 h and cell proliferation was evaluated using a CCK8 solution kit. Cell viability was determined using scanning with a microplate reader at 450 nm. Data were expressed as the percentage of viable cells as follows: relative viability (%) = [A450(treated) − A450(blank)]/[A450(control) − A450(blank)] × 100%.

WESTERN BLOT ANALYSIS

Total protein was isolated from cells using Cell Extraction Buffer (Biosource, Camarillo, CA) supplemented with protease and phosphatase inhibitors and precleared using centrifugation, followed by measuring protein concentrations using the BCA Protein Assay kit (Pierce, Rockford, IL). The following primary antibodies were used in this study, E-cadherin (Cat. No. 3195), Vimentin (Cat. No. 3932), Snail (Cat. No. 3879), Notch-2 (Cat. No. 4530), Notch-3 (Cat. No. 2889), Notch-4 (Cat. No. 2423), Jagged-2 (Cat. No. 2205), Dll-4 (Cat. No. 2589), cyclin D1 (Cat. No. 2922), p21 Waf1/Cip1 (Cat. No. 2947), β-actin (Cat. No. 4967), EGFR (Cat. No. 4267), pEGFR (Cat. No. 2236), AKT (Cat. No. 2967), pAKT (Cat. No. 4058), Erk1/2 (Cat. No. 4695), and pErk1/2 (Cat. No. 4370) were purchased from Cell Signaling Technology. Notch-1 (Cat. No. ab8925), Dll-1 (Cat. No. ab76655), and Dll-3 (Cat. No. ab103102) were ordered from Abcam, Inc. Jagged-1 (Cat. No. sc-6011) and Hes-1 (Cat. No. sc-25392) were purchased from Santa Cruz Biotechnology. Equal protein loading was confirmed with probing for β-actin expression. Appropriate secondary antibodies conjugated to horseradish peroxidase were used, including anti-mouse or anti-rabbit IgG (GE Healthcare). Proteins were visualized by Amersham enhanced chemiluminescence (GE Healthcare). Developed films were digitized with a Microtek scanner. Protein content was quantified using scanning densitometry, with ImageJ software (National Institutes of Health, Bethesda, MD). Preliminary studies demonstrated that there was a linear relationship between densitometry measurements and the amounts of protein loaded onto the gels.

REAL-TIME REVERSE TRANSCRIPTION-PCR (qPCR) ANALYSIS

Total RNA was extracted from the cells (1×10^6 cells) using Trizol plus kit (TaKaRa, Japan). First-strand cDNA synthesis was performed using Promega kit. Synthesized cDNA was used for qPCR analysis using Lightcycler (Roche, Switzerland) based on the manufacturer's instructions. β-Actin was used as an internal control. All samples were amplified in duplicates. Relative changes in the amount of transcripts in each sample were determined with β-actin normalization of mRNA levels.

IMMUNOFLUORESCENCE ASSAY

Cytospin preparations of the cells (5×10^5 cells/ml) were obtained on glass slides pretreated with poly-D-lysine using centrifugation at 600 rpm for 3 min in a cytocentrifuge. Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized in 0.5% Triton X-100 for an additional 10 min, followed by incubation with the blocking solution (10% goat serum in PBS). Afterwards, cells were incubated with anti-E-Cadherin (Cat. No. 3195, Cell Signaling Technology), anti-Vimentin (Cat. No. 3932, Cell Signaling Technology), anti-Snail (Cat. No. 28199, Santa Cruz Biotechnology) for 2 h or overnight, washed and incubated with secondary antibodies (Invitrogen) and DAPI (Sigma) for an additional 2 h, followed by mounting with slow fade Light Anti fade Kit (Invitrogen). Cell and microvessel density counts were performed using ImageJ software, compiled, and analyzed under ×200 magnification. The results were representative of two independent experiments.

PLASMIDS AND PLASMID CONSTRUCTION

The pcDNA-HA-N1IC expression construct was kindly provided by Yeh TS (National Yang-Ming University) and contained cDNA encoding the human Notch1 receptor intracellular domain with an N-terminal hemagglutinin (HA) tag [Yeh et al., 2003]. PC9 cells were stably transfected with N1IC or vector alone (pcDNA3) and maintained under Geneticin (G418, Invitrogen) selection. PC9/AB2 cells were transiently transfected with Notch-1 siRNA (Cat. No. sc-36095, Santa Cruz Biotechnology) and control siRNA (Cat. No. sc-44236, Santa Cruz Biotechnology), respectively using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells transfected with Notch-1 siRNA were noted as siNotch-1 and the cells with control siRNA were named siControl.

ANOIKIS ASSAY

PC9/AB2 cells transfected with control or Notch-1 siRNAs were cultured in dishes coated with poly-2-hydroxyethyl methacrylate (polyHEMA, Sigma) to prevent them from adhering to the plastic dishes. Control or siRNA-transfected cells were grown to 70% confluence and tripsinized, and then plated at a density of 2.5×10^5 cells per 60 mm polyHEMA dishes (polyHEMA dished were constructed by applying 2 ml of 10 mg/ml solution of polyHEMA in ethanol onto the dish, drying in tissue culture hood). Cells were harvested and incubated at 37°C with 0.25% trypsin for 1 min to prevent cell aggregation at the indicated time. After 72 h in suspension, cells were collected, washed, and dispersed using trypsinization. Trypan blue exclusion assay was performed in duplicate and the results represent average values from three independent experiments.

TUNEL ASSAY

Percentage of apoptotic cells was determined using the TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling). Cytospin preparations of PC9/AB2 cells transfected with control or Notch-1 siRNAs were stained with Takara in situ apoptosis detection kit (Takara, Japan). Briefly, cells were fixed with 4% paraformaldehyde solution for 30 min, followed by treatment with 0.3% H_2O_2–methanol solution and with permeabilization buffer for 2 min on ice. Subsequently, cells were incubated with terminal deoxynucleotidyl transferase (TdT) enzyme and anti-FTIC-HRP. For each slide, three randomly selected microscopic fields were observed at ×400 magnification and 100 cells/fields were evaluated.

SOFT AGAR COLONY FORMATION ASSAY

PC9/AB2 cells (1×10^4 cells) transfected with control or Notch-1 siRNAs were suspended with 2.5 ml of 0.4% low-melting point agarose (Cat No. 5010, Biowhittaker Molecular Application) in RPMI1640 containing 10% FBS. The upper layer was seeded onto 60 mm dishes coated with 0.5% low-melting-point agarose in RPMI1640 with 10% FBS. Medium was changed every 3 days for 2 weeks. The number of colonies that were 200 μM or larger were counted following 2 weeks.

STATISTICAL ANALYSES

Experiments presented are representative of three or more repeated trials. Data are presented as the mean ± SEM. Comparisons between groups were evaluated using a two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

PC9/AB2 CELLS SHOW SIGNIFICANT RESISTANCE TO GEFITINIB

A CCK-8 kit was utilized to assess the proliferative ability of PC9 and PC9/AB2 cells. Growth rate in PC9 cells is faster than in PC9/AB2 cells ($P < 0.05$) (Fig. 1A). Gefitinib had little effect on cell proliferation in PC9/AB2 cells. Values for IC50 of gefitinib in PC9/AB2 cells was 25.11 ± 2.25 μmol/L, 322 times greater than that in PC9 cells (0.078 ± 0.001 μmol/L, $P < 0.001$) (Fig. 1B). Resistance in PC9/AB2 cells was maintained for 1 year when they were maintained in gefitinib-free medium. Expression of EGFR, Akt and Erk1/2 was detected using Western blot. We found that Gefitinib (2 μmol/L) significantly decreased the phosphorylation of EGFR, Akt and Erk1/2 in PC9 cells, but not in PC9/AB2 cells (Fig. 1C,D). Note that the total protein levels of EGFR, Akt, and Erk1/2 had no effect.

PC/AB2 CELLS DISPLAY MORPHOLOGIC CHANGES CONSISTENT WITH EMT

As shown in Figure 2A, PC/AB2 cells displayed elongated, irregular fibroblastoid morphology. In contrast, PC9 cells were rounded in shape, typical of an epithelial appearance. These changes in phenotype suggested that PC/AB2 cells underwent EMT. To further confirm this, we determined the expression of markers of epithelial and mesenchymal phenotypes. We observed that expression of E-cadherin protein was greatly reduced in PC/AB2 cells (Fig. 2B,C). However, elevated levels of Vimentin and Snail were observed in PC/

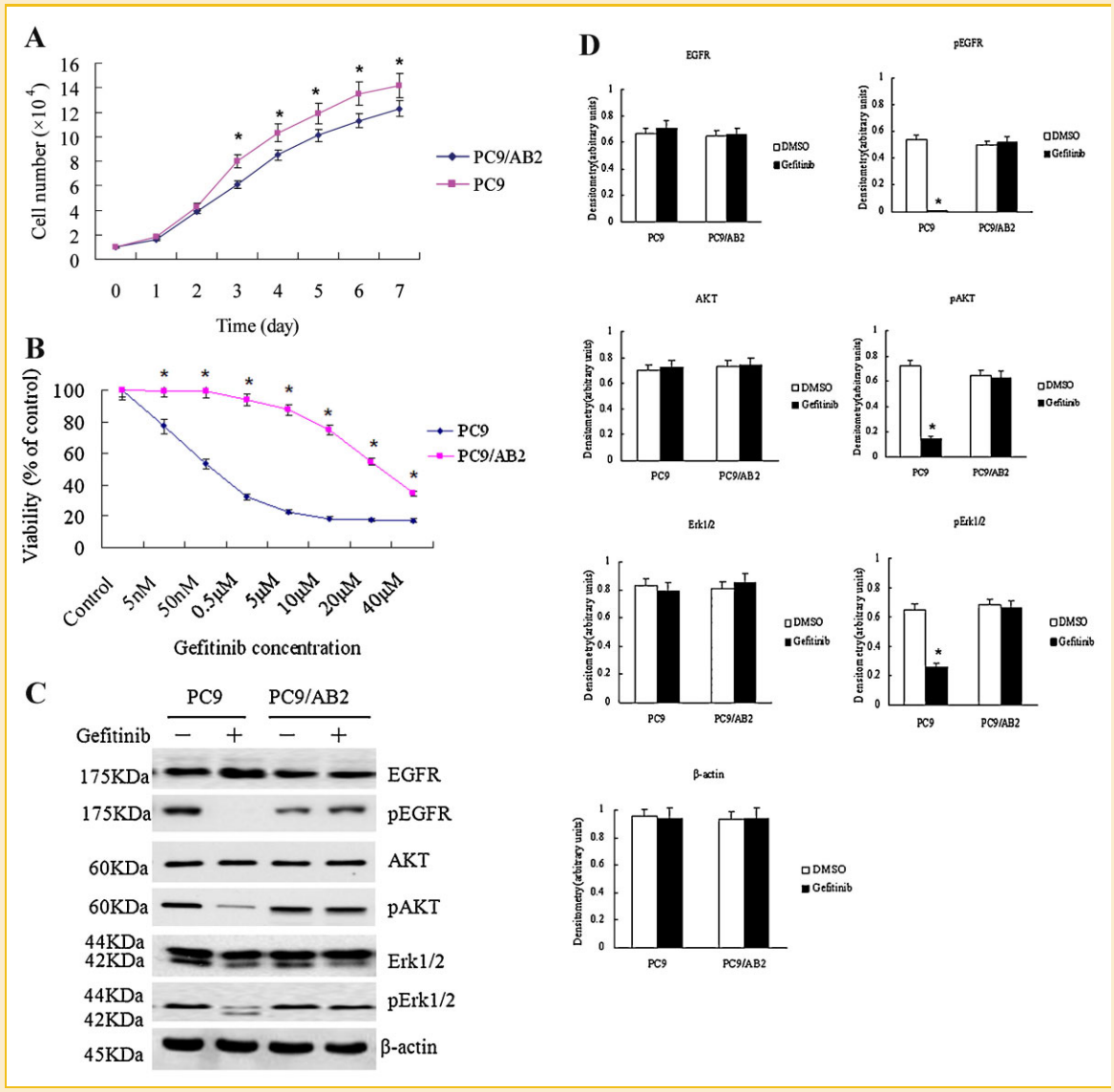

Fig. 1. Suppression of proliferation in PC9 and PC9/AB2 cells by gefitinib. A: PC9 and PC9/AB2 cells were seeded onto 96-well plates at an initial density of 5×10^3 cells/well for 24 h and cultured with increased concentrations of gefitinib for an additional 48 h. Afterwards, cell proliferation of PC9 cells and PC9/AB2 cells was determined using a CCK-8 kit. Bars indicated means ± standard deviation of three independent experiments. Asterisk (*) indicates significant difference as compared to PC9 cells ($P < 0.05$). B: PC9 and PC9/AB2 cells (5×103 per well) were seeded onto 96-well plates in triplicate and treated with increasing concentrations of gefitinib as indicated for 48 h. Afterwards, their viability was measured using the CCK-8 assay and plotted as a percentage of the viability of untreated cells (control). Values of IC50 for gefitinib in PC9 and PC9/AB2 cells were 0.078 ± 0.001 and 25.11 ± 2.25 μmol/L, respectively. Asterisk (*) indicates significant differences as compared to the control untreated cells ($P < 0.01$). C: Western blot analysis of changes of downstream of EGFR signaling pathway in PC9 and PC9/AB2 cells pre- and post-treatment of 2 μmol/L gefitinib. D: Densitometric quantification of (C). Asterisk (*) indicates significant differences as compared to control ($P < 0.05$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

AB2 cells, which were consistent with the EMT process and suggested that acquisition of EMT phenotype in gefitinib-acquired resistant PC9 cells. As expected, similar results were observed in mRNA expression as determined by real-time RT-PCR (Fig. 2D).

To further examine whether PC9/AB2 cells are EMT-like cells, we assessed the expression of E-cadherin, Vimentin, and Snail using immunofluorescence staining following cytospin. We found that Vimentin and Snail are highly expressed in PC9/AB2 cells, whereas the expression of E-cadherin was reduced in PC9/AB2 cells compared with that in PC9 cells ($P < 0.05$) (Fig. 3). Having confirmed that PC9/AB2 cells acquired the EMT phenotype, we next investigated the role of Notch-1 signaling in EMT.

NOTCH-1 SIGNALING AND ITS TARGET GENE ARE INVOLVED IN THE EMT IN PC9/AB2 CELLS

To explore whether the Notch-1 signaling pathway is involved in EMT in PC/AB2 cells, we examined the levels of Notch1–4 receptors, ligands and the Notch target gene Hes-1 at mRNA and protein levels using qPCR and Western blot, respectively. Protein expression of Notch1–4 intracellular domain (ICD), the transcriptional active

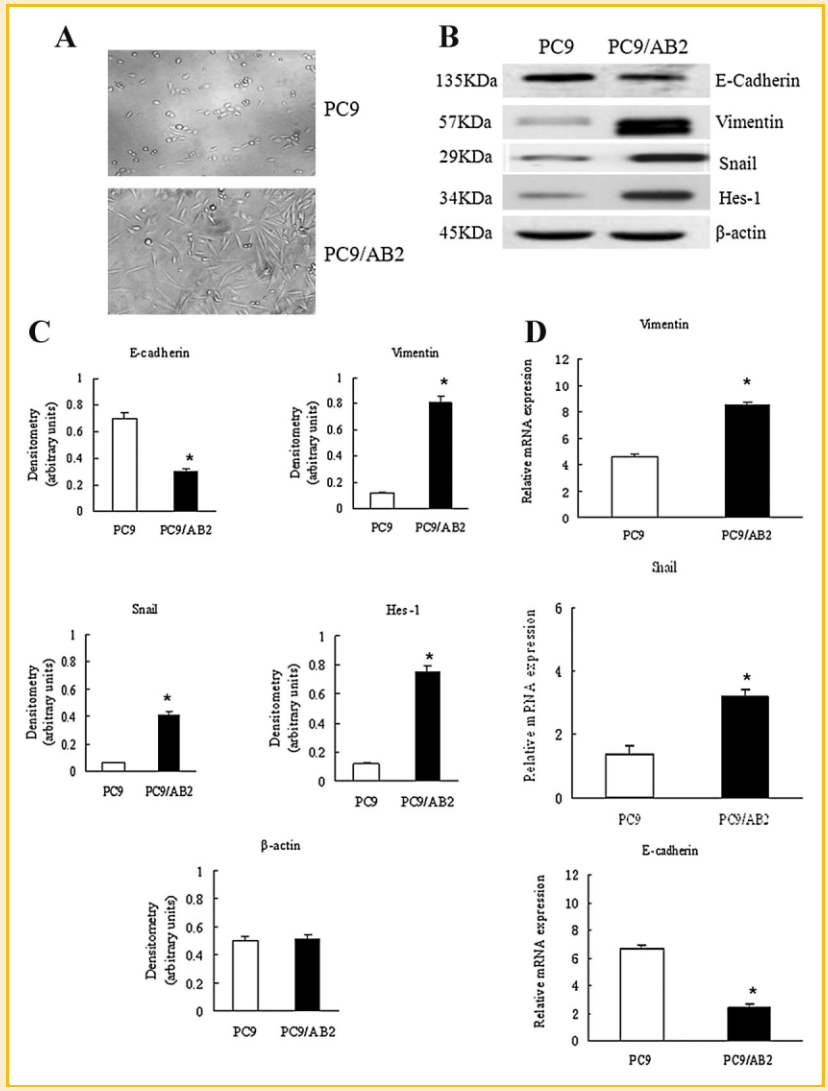

Fig. 2. EMT phenotype changes in PC9/AB2 and PC9 cells. A: Phase-contrast images of PC9/AB2 and PC cells, respectively. B: Total protein was isolated from PC9/AB2 and PC cells and Western blot analyses were performed to detect the expression of Vimentin, Snail, and E-cadherin proteins. β-Actin was used as the loading control. C: Densitometric quantification of (B). Asterisk (*) indicates significant differences ($P < 0.05$). D: Total RNA was isolated and qPCR was performed to quantify mRNA expression of E-cadherin, Vimentin, and Snail in PC9/AB2 and PC9 cells. Values were expressed as relative arbitrary units following normalization with β-actin mRNA expression. Asterisk (*) indicates significant differences as compared to PC9 cells ($P < 0.05$).

portion of the Notch receptors were detected using Western blot. Notch ICD is released from the plasma membrane and translocates into the nucleus, where it forms a nuclear complex with the DNA-binding protein CSL (CBF1, Sel, Lag-1). This interaction leads to transcription of the bHLH factors, Hairy-enhancer of Split (Hes) and related genes Hey [Wang et al., 2011]. PC9/AB2 cells had an increased expression of Notch-1 mRNA and protein levels. However, there was no significant difference between expression of other Notch receptors and ligands in PC9 and PC9/AB2 cells (Fig. 4A–E).

We next examined whether Notch target gene, Hes-1, was affected by EMT in PC9/AB2 cells. We found that Hes-1 was up-regulated in PC9/AB2 cells (Fig. 4F). We next tested whether cyclinD1 and p21 Waf1/Cip1 were also correlated with the acquisition of EMT characteristics in PC9/AB2 cells. Our results showed that mRNA and protein levels of cyclinD1 were increased, while reducing p21 Waf1/Cip1 protein level was observed in PC9/AB2 cells (Fig. 4F–H). These results suggested that upregulation of Hes-1 was consistent with activation of the Notch-1 signal; therefore, canonical Notch1 signaling pathway was involved in EMT.

NOTCH-1 ACTIVATION IS REQUIRED FOR EMT IN LUNG CANCER CELLS IN VITRO AND CONTRIBUTES TO GEFITINIB RESISTANCE

To further explore the role of Notch-1 signaling in the acquisition of EMT, we exogenously transfected a vector containing the Notch-1 receptor intracellular domain (N1IC), the activated form of the Notch-1 receptor, into PC9 cells. The result showed that exogenous expression of N1IC considerably enhanced the expression of Vimentin and Snail proteins, whereas the expression of E-cadherin proteins was attenuated in PC9/HA-N1IC cells (Fig. 5A,B). These

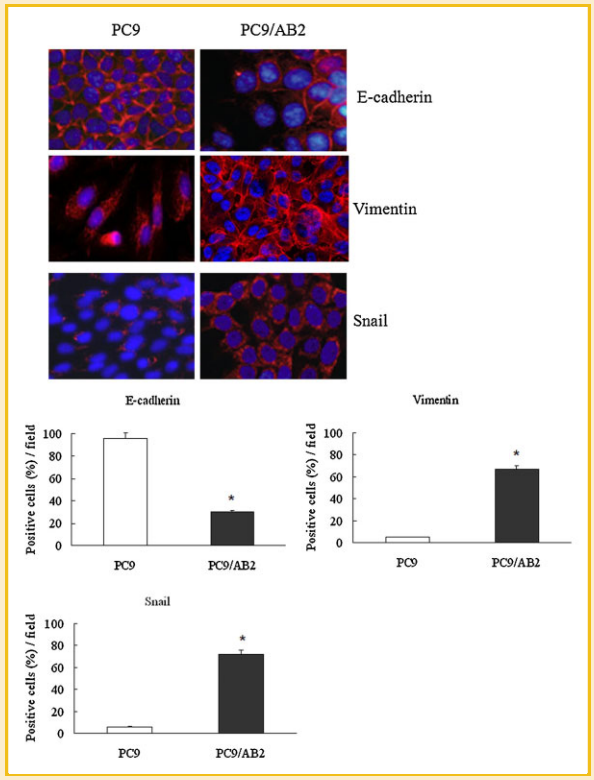

Fig. 3. Expression of E-cadherin, Vimentin, and Snail in PC9 and PC9/AB2 cells. PC9 and PC9/AB2 cells were prepared and fixed on microscope slides using cytospin centrifugation. Expression of E-cadherin, Vimentin, and Snail (red) in PC9 and PC9/AB2 cells were determined using immunofluorescence and confocal microscopy. Nuclei were stained with DAPI (blue). Quantification of immunocytostaining positive cells was performed using Image J software. Every section under analysis was a minimum distance of 150 μm from the next, and a total of six randomly selected fields for analysis. Asterisk (*) indicates significance difference as compared to PC9 cells ($P < 0.05$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

findings were correlated with those of the PC9 acquired resistance cells (PC9/AB2) with Notch-1 overexpression. Values of IC50 for gefitinib in PC9/pcDNA3 and PC9/HA-N1IC cells were 0.079 ± 0.003 and 19.51 ± 1.15 μmol/L, respectively ($P < 0.01$) (Fig. 5C). Together, these results suggested that activation of Notch-1 signaling strongly promoted EMT and attenuated gefitinib sensitivity in PC9 cells. On the contrary, silencing of Notch-1 using siRNA inhibited not only Notch-1, but also Hes-1 and cyclinD1 proteins (Fig. 6B,C). Moreover, we found that PC9/AB2 cells transfected with Notch-1 siRNA displayed a round cell-like morphology 72 h after Notch-1 siRNA transfection (Fig. 6A). Western blot showed that the expression of E-cadherin was significantly increased; suggesting down-regulation of Notch-1 expression in PC9/AB2 cells resulted in reversal of the EMT phenotype (Fig. 6D,E). In addition, we found a significant reduction in the expression of Snail and Vimentin in Notch-1 siRNA transfected cells. A CCK-8 kit was also used to assess the proliferative ability of PC9/AB2 cells transfected with siNotch-1. Growth rate in PC9/AB2 cells knockdown of Notch-1 is significantly lower than that in PC9/AB2 cells with siControl ($P < 0.05$) (Fig. 6F).

SILENCING OF NOTCH-1 DECREASED ANOIKIS AND COLONY-FORMATION, BUT ENHANCED SENSITIVITY OF GEFITINIB IN PC9/AB2 CELLS

PolyHEMA prevents cells from attaching to the culture dishes and forces the cells to grow in suspension [Munoz et al., 2010]. Cells form cell aggregates to resist apoptosis [Daubriac et al., 2009]. The size and tightness of the aggregates vary depending on the cell lines used. We found that more and larger cell aggregations in PC9/AB2 cells than in PC9 cells under suspension culture. More viability were found in PC/AB2 cells ($52 \pm 3 \times 10^4$) than in PC9 cells ($29 \pm 2 \times 10^4$) after 72 h suspension ($P < 0.05$) (Fig. 7A,B). PC9/AB2 cells transfected with Notch-1 siRNA formed small and loose aggregates, while PC9/AB2 cells transfected with control siRNA formed large and compact aggregates (Fig. 7C,D). Trypan blue exclusion assay results showed that viability of PC9/AB2 cells transfected with Notch-1 siRNA and control siRNA were $32 \pm 2 \times 10^4$ and $47 \pm 3 \times 10^4$, respectively ($P < 0.05$). TUNEL assay showed that $26.5 \pm 1.7\%$ of PC9/AB2 cells transfected with control siRNA displayed positive TUNEL staining, whereas $42.1 \pm 3.5\%$ of PC9/AB2 cells transfected with Notch-1 siRNA showed apoptotic characteristics ($P = 0.003$). (Fig. 7E,F). Colony-forming activity of PC9/AB2 cells transfected with Notch-1 siRNA demonstrated reduced anchorage-independent cell growth, resulting in significantly less and smaller colonies (52 ± 3 colonies/10 fields), whereas PC9/AB2 cells transfected with control siRNA resulted in bigger and more abundant colonies (67 ± 4 colonies/10 fields) ($P < 0.05$) (Fig. 8A,B).

Since Notch-1 expression was involved in the EMT phenotype in gefitinib acquired resistance PC9 cells, we then examined if down-regulation of Notch-1 expression could affect the sensitivity of PC9/AB2 cells to gefitinib. We showed that Notch-1 siRNA significantly enhanced gefitinib sensitivity in PC9/AB2 cells (Fig. 8C). Values of IC50 for gefitinib in PC9/AB2 cells transfected with siControl or siNotch-1, treated with DAPT and PC9 parental cells were 21.861 ± 3.150, 0.211 ± 0.021, 0.561 ± 0.326, and 0.079 ± 0.003 μmol/L, respectively. Both knockdown of Notch-1 and treatment of DAPT in PC9/AB2 cells increased sensitivity of gefitinib when comparing to PC9/AB2 cells transfected with control siRNA or Notch-1 siRNA ($P < 0.05$). This was also found in PC9/AB2 cells treated with DAPT when comparing to PC9/AB2 parental cells ($P < 0.05$).

DISCUSSION

Studies have shown that EMT is associated with drug resistance and cancer cell metastasis [Li and Zhou, 2011]. It is now widely accepted that epithelial cells can acquire a mesenchymal phenotype through complex processes. Strong multiple gene signatures indicative of EMT were identified as determinants of insensitivity to EGFR-TKI gefitinib through completed gene expression [Uramoto et al., 2011]. This observation was further supported through analysis of classic EMT marker proteins, including E-cadherin and Vimentin. In this study, we used gefitinib sensitive PC9 cell line and a gefitinib

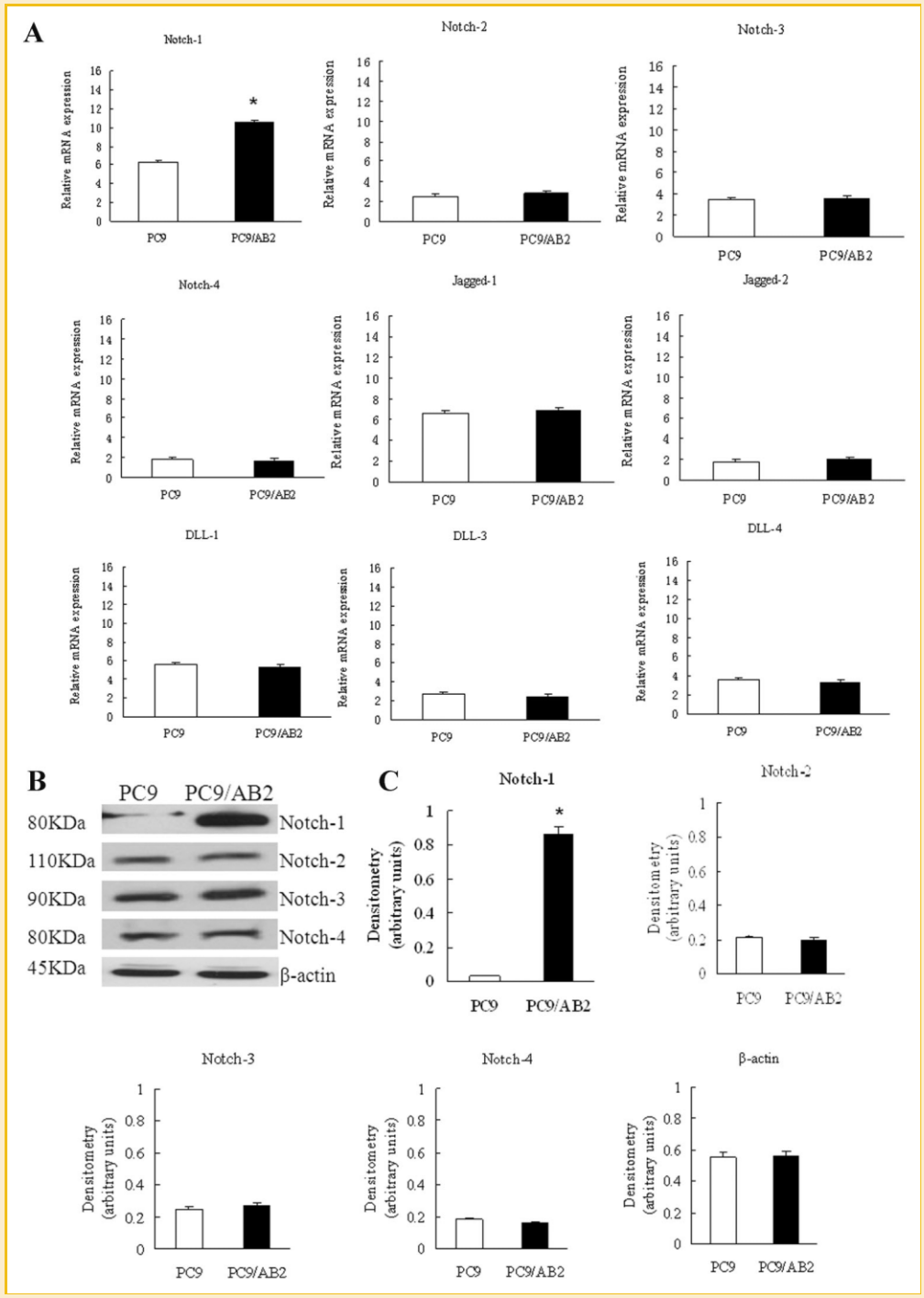

Fig. 4. The expression of Notch receptors, ligands, and downstream targets in PC9 and PC9/AB2 cells. A: Total RNA were isolated from PC9 and PC9/AB2 cells and qPCR was used to determine mRNA levels of Notch receptors and ligands. Asterisk (*) indicates significant differences compared to PC9 cells ($P < 0.05$). B: Cellular protein was isolated from cells and Western blot was performed to detect protein expression of Notch receptors. C: Densitometric quantification of (B). Asterisk (*) indicates significant differences compared to PC9 cells ($P < 0.05$). D: Western blot was performed to detect protein expression of Notch ligands. E: Densitometric quantification of (D). F: Total RNA was isolated from PC9 and PC9/AB2 cells and qPCR was used to measure Hes-1, P21 Waf1/Cipl, and cyclinD1 mRNA levels. Hes-1 and cyclin D1 were upregulated in PC9/AB2 cells. P21 Waf1/Cipl was downregulated in PC9/AB2 cells. Asterisk (*) indicates significant difference compared to PC9 cells ($P < 0.05$). G: Cellular protein was isolated from cells and Western Blot was performed to detect Hes-1, P21 Waf1/Cipl, and cyclinD1 protein expression. β-Actin was used as the loading control. H: Densitometric quantification of (G). Asterisk (*) indicates significant differences compared to PC9 cells ($P < 0.05$).

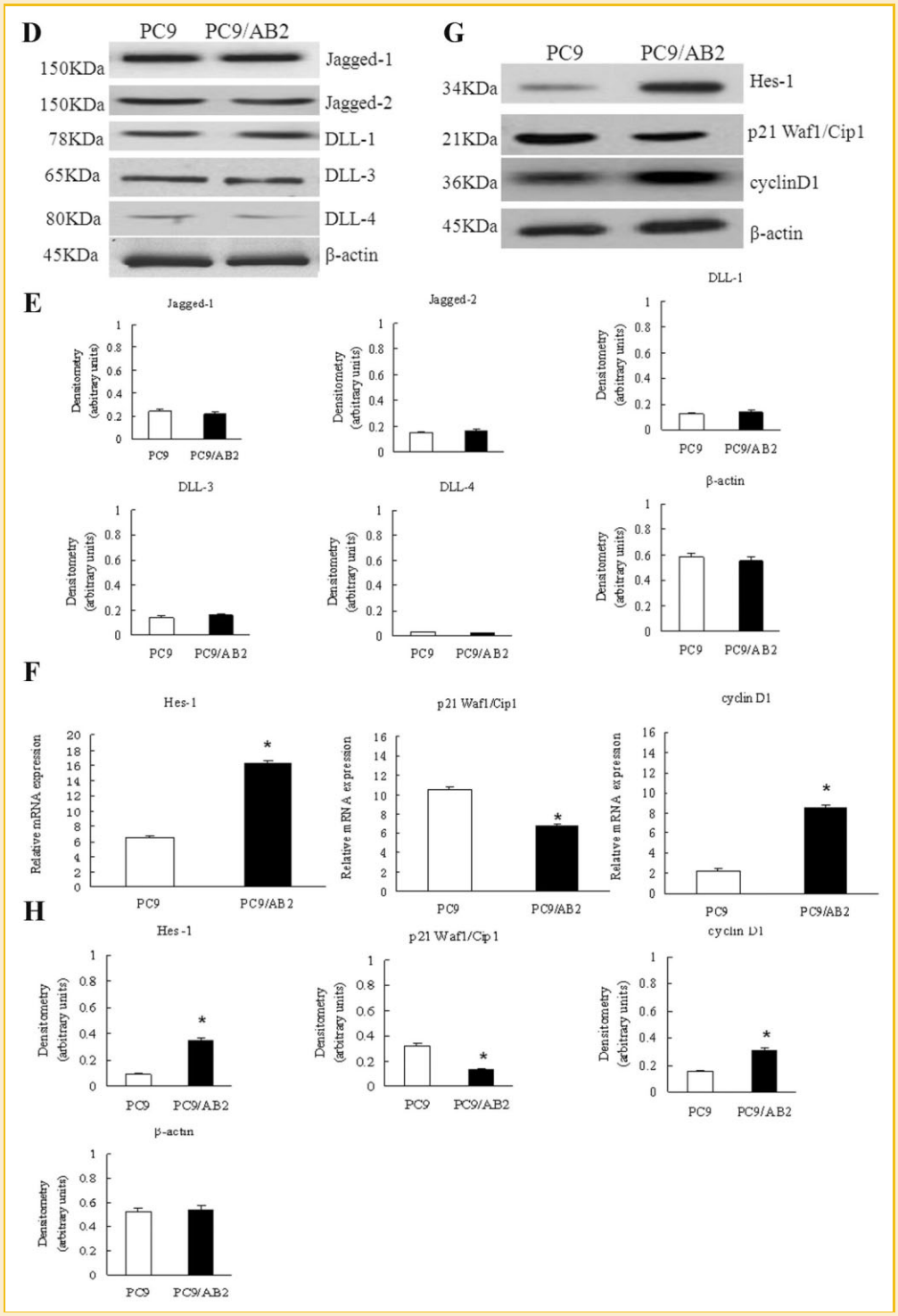

Fig. 4. (*Continued*)

resistant PC/AB2 cell line to investigate the molecular mechanisms of resistance and cellular behaviors involved. Genotypes of EGFR were determined in PC9 and PC9/AB2 cells. Both PC9 and PC9/AB2 harbored only exon 19 deletions (15 bp deletion) without other EGFR mutations [Ju et al., 2010]. PC/AB2 cells displayed elongated, irregular fibroblastoid morphology. In contrast, PC9 cells had a rounded shape, typical of epithelial cells. These changes in phenotype suggest that PC9/AB2 cells underwent EMT. Results from qPCR, Western blot, and immunofluorescence confirmed the phenotype of EMT in PC9/AB2 cells. Wang et al. [2011] showed that Notch-2 and its ligand, Jagged-1, were highly up-regulated in Gemcitabine resistant cells, which was consistent with the role of the

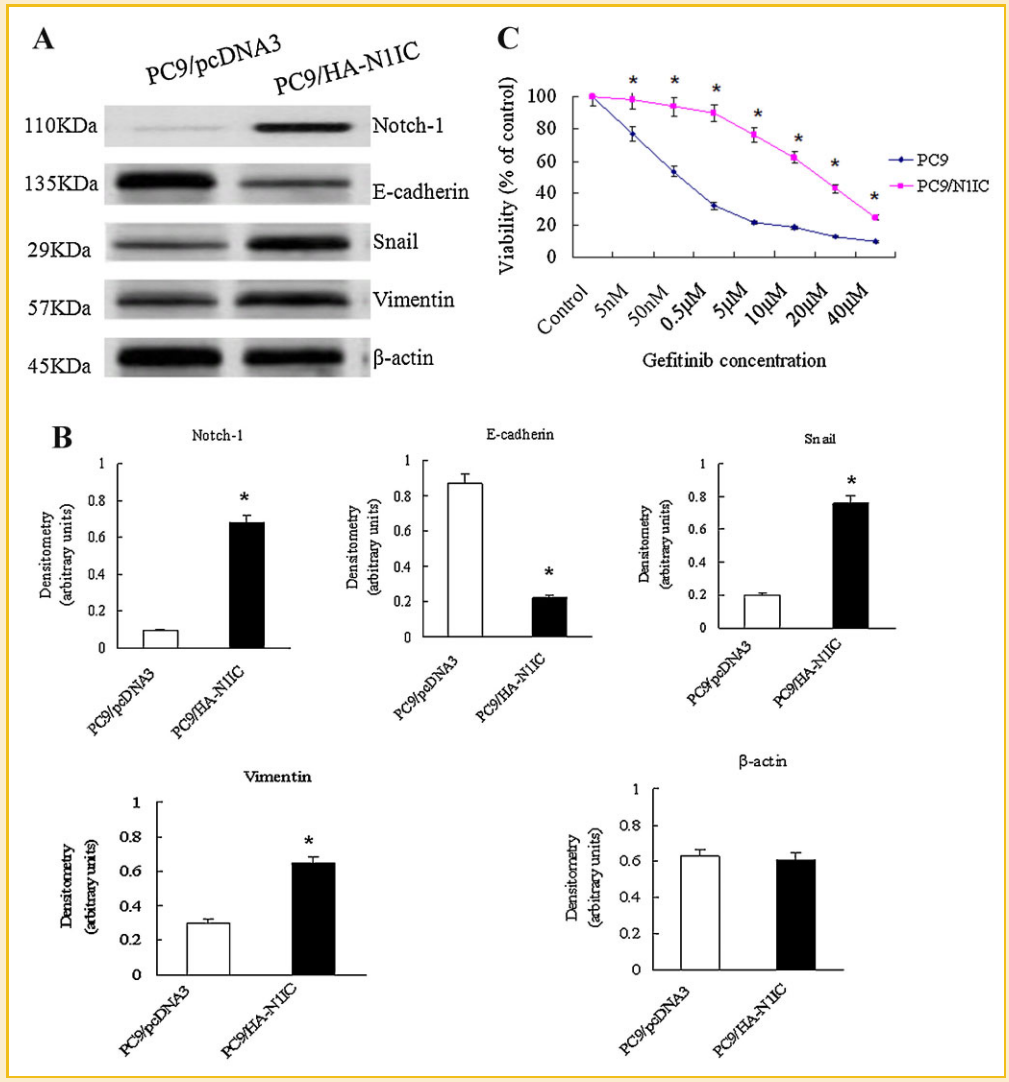

Fig. 5. Notch-1 promotes the EMT phenotype. A: PC9 cells were transfected with control vector (pcDNA-HA) or N1IC-expressing construct (pcDNA-HA-N1IC) for 24 h. Western blot was then performed to determine the protein levels of Notch-1, E-cadherin, Snail, and Vimentin in PC9/pcDNA3 and PC9/HA-N1IC cells. β-Actin was used as the loading control. B: Densitometric quantification of (A). Asterisk (*) indicates significant differences compared to control cells ($P < 0.05$). C: The effect of gefitinib on cell viability. PC9/pcDNA3 and PC9/HA-N1IC cells (5×10^3 per well) were seeded on 96-well plates in triplicate and treated with increasing concentration of gefitinib as indicated for 48 h. Afterwards, their viability was measured with a CCK-8 assay and plotted as a percentage of the viability of untreated cells (control). Values of IC50 for gefitinib in PC9/pcDNA3 and PC9/HA-N1IC cells were 0.079 ± 0.003 and 19.51 ± 1.15 μmol/L, respectively. D: Asterisk (*) indicates significance difference as compared to the control untreated cells ($P < 0.01$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Notch signaling pathway in the acquisition of EMT and cancer stem-like cell phenotype.

Activation of Notch signaling is known to regulate the expression of several target genes. One study showed that Notch signaling was essential to control cell development and differentiation and played important roles in regulation of proliferation and apoptosis [Pannuti et al., 2010]. Another study found that Notch-mediated EMT was associated with increased expression of the Snail transcription factor [Saad et al., 2010]. Inhibition of Notch-1 activation by the gamma-secretase inhibitor DAPT during EMT attenuated the decrease in E-cadherin and Snail and increase in alpha-SMA, MMP-2 and -9 expressions [Pannuti et al., 2010]. In addition, the Notch-1 pathway was found to up-regulate Snail expression by recruitment of the intracellular Notch-1 to the Snail promoter [Foubert et al., 2010]. Consistent with these findings, our results showed that increased activation of Notch-1 resulted in increased expression of target gene Hes-1 and decreased expression of E-cadherin in PC9/AB2 cells. In addition, we found that stable expression of the active form of Notch-1(N1IC) resulted in increased expression of Vimentin and Snail, while decreasing expression of E-cadherin in PC9/HA-N1IC cells. This further implicated a critical role for Notch-1 in this process. On the contrary, down-regulation of Notch-1 by siRNA led to reversal of the EMT phenotype in PC9/AB2 cells. Taken together, these results suggest that activation of Notch-1 could enhance the induction of EMT phenotype during the acquisition of resistance in PC9 cells.

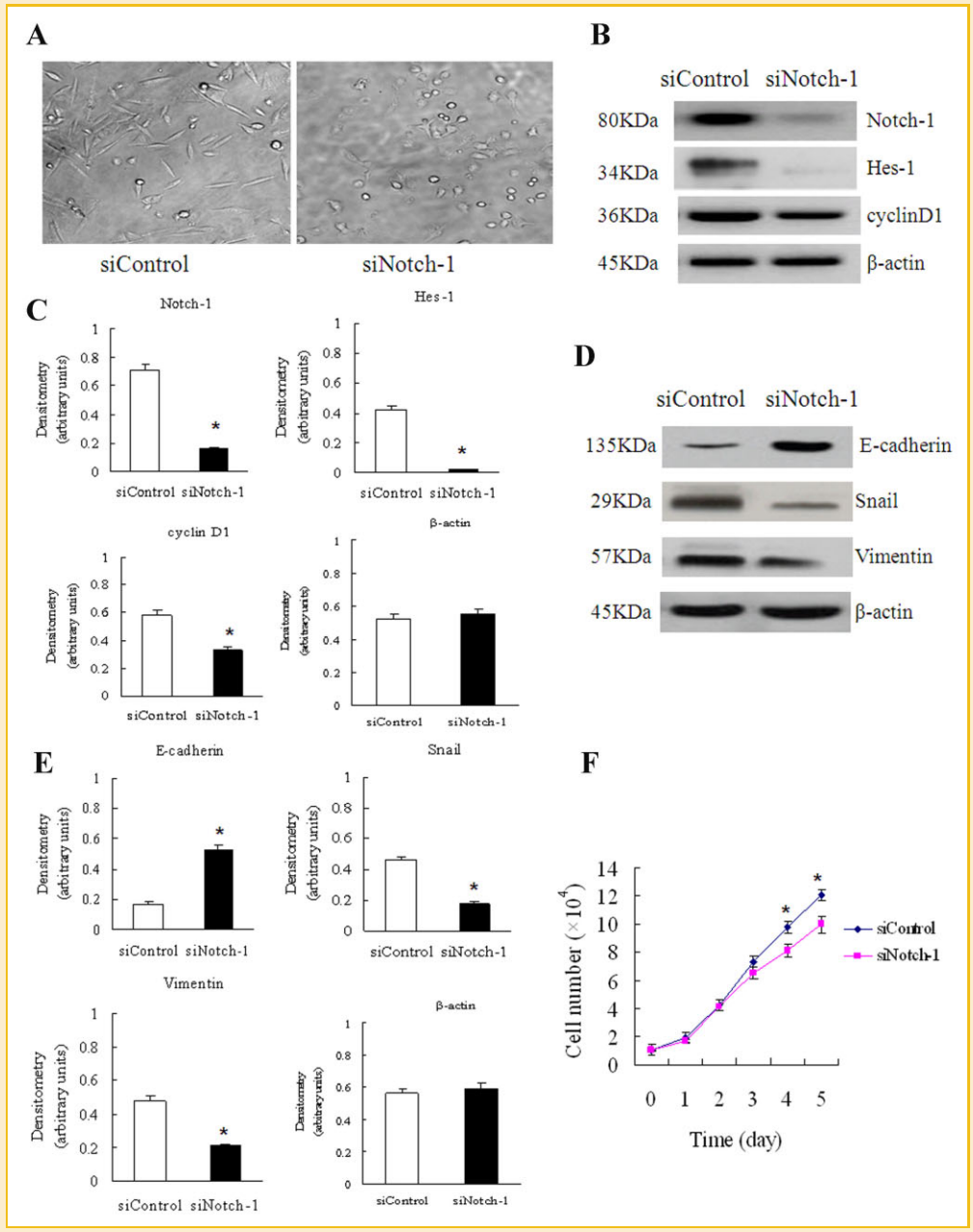

Fig. 6. The Notch-1 pathway contributes to the regulation of molecular markers of EMT in PC9/AB2 cells. A: Phase-contrast images of PC9/AB2 cells transfected with control (siControl) or Notch-1 siRNA (siNotch-1) for 24 h. Down-regulation of Notch-1 with siRNA could cause reversal of EMT phenotype of PC9/AB2 cells. B: PC9/AB2 cells were transfected with siControl or siNotch-1 for 24 h, followed by Western blot for detecting the protein expression of Notch-1, Hes-1, and cyclinD1. C: Densitometric quantification of (B). Asterisk (*) indicates significant differences as compared to control cells ($P < 0.05$). D: PC9/AB2 cells were transfected with siControl or siNotch-1 for 24 h. Afterwards, Western blot was performed to detect the markers of EMT phenotypes. E: Densitometric quantification of (D). Asterisk (*) indicates significant differences as compared to control cells ($P < 0.05$). F: Cell proliferation of PC9/AB2 cells transfected with siControl and siNotch-1 was determined by CCK-8 kit. Bars indicated means ± standard deviation of three independent experiments. Asterisk (*) indicates significant difference as compared to the control cells ($P < 0.05$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

In our experiment, the expression of cyclinD1 in PC9/AB2 cells is significantly higher than that in PC9 cells. Meanwhile, knockdown of Notch-1 decreased cyclinD1 expression in PC9/AB2 cells. Cyclin D1 is a target of beta-catenin-dependent Wnt signaling. Notch1 expression is controlled by beta-catenin-dependent Wnt signaling. This effect is partly mediated by cyclin D1 that influences histone modification of Notch-1 DNA [Bienvenu et al., 2010]. Also, there is increasing evidence showed that p21 Waf1/Cip1 is a regulator of cyclin D1. Besides the Hes and Hey family, other Notch target genes have been identified, including c-myc, cyclin D1, and p21 Waf1/Cipl [Rangarajan et al., 2001]. Castella et al. [2000] identified the promoter of the cyclin-CDK inhibitor, p21, as a direct target for Hes-1-mediated transcriptional repression in the inducible PC12 cells. Expression of Hes-1 strongly repressed transcription of the p21

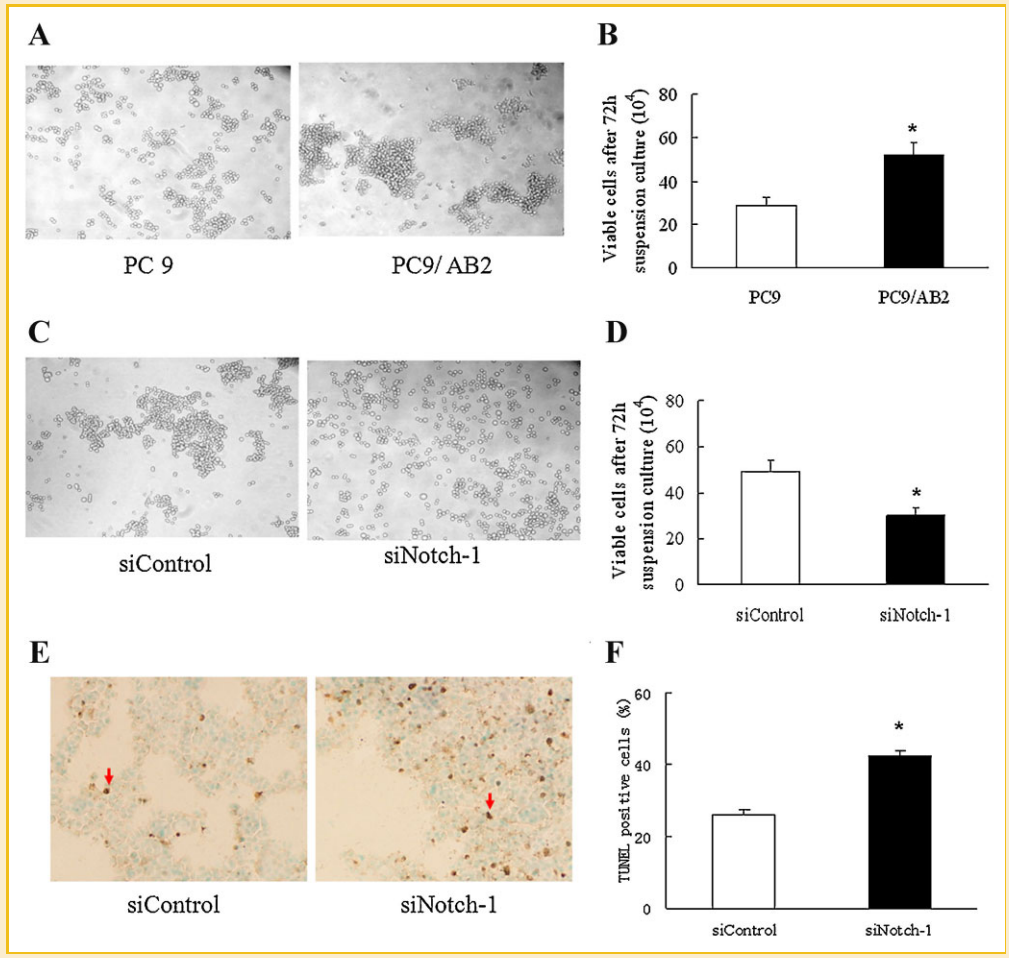

Fig. 7. Silencing of Notch-1 inhibits anoikis and induces apoptosis in PC9/AB2 cells. A: Cells were cultured in dishes coated with polyHEMA for up to 72 h. Afterwards, aggregation was assessed. More and larger cell aggregations in PC9/AB2 cells than in PC9 cells were found. B: Trypan blue exclusion assay was conducted to count cell viability of PC9 cells and PC9/AB2 cells. The data were presented as mean ± SEM of triplicate determinations from three independent experiments. Notch-1 siRNA inhibited anoikis and viable cells reduced. Asterisk (*) indicates a significant difference between PC9 cells and PC9/AB2 cells ($P < 0.05$). C: Aggregation of Notch-1 siRNA PC9/AB2 cells was inhibited by avoiding initiation of anoikis. D: Trypan blue exclusion assay was conducted to determine cell viability. Notch-1 siRNA inhibited anoikis and viable cells were reduced. Asterisk (*) indicates a significant difference compared to control siRNA treated cells ($P < 0.05$). E: After culturing for 72 h, cells were fixed on microscope slides using cytospin centrifugation and anoikis was assessed using the TUNEL assay with Takara in situ apoptosis detection kit. TUNEL positive cells (nucleus brown stained, arrow) were considered as apoptotic cells. F: Three randomly selected microscopic fields were observed at ×400 magnification and at least 100 cells/fields were evaluated. Data were presented as mean ± SEM of triplicate determinations from two independent experiments. Asterisk (*) indicates significant differences compared to CS control cells ($P < 0.05$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Waf1/Cipl promoter. Nakamura et al. [2008] found that ratios of corneal epithelial stem/progenitor cells positive for cyclin D1, a G1-specific marker, were increased when Hes-1 was infected, suggesting that persistent Hes-1 expression prolongs the G1 phase. These findings, together with ours, indicate that p21 Waf1/Cip1 and cyclin D1 are not only direct Notch targets but also regulated by the prominent target of Notch signaling Hes-1. We found that cyclinD1 expression was significantly increased in PC9/AB2 cells where Notch-1 signaling was activated. CyclinD1 is not only the marker for invasion of cancer cells, but also critical for cell cycle progression of EMT cells [Zhang et al., 2010]. Also, increasing evidence has shown that p21 Waf1/Cipl is a positive regulator of cyclin D1. P21 Waf1/Cipl promotes the nuclear accumulation of cyclin D1 complexes via inhibition of cyclin D1 nuclear export [Liu et al., 2009]. We showed that PC9/AB2 cells had lower expression of p21 Waf1/Cipl when Notch-1 is highly activated. Therefore, we hypothesized that PC9/AB2 cells could have potential aggressive behavior partly due to the upregulation of cyclinD1 and downregulation of p21 Waf1/Cipl during the acquisition of the EMT phenotype. EMT has been linked to tumor progression, wherein we found that transfection of PC9/AB2 cells with Notch-1 siRNA significantly inhibited resistance to anoikis as well as anchorage independent cell growth. These results were consistent with the mechanistic role of Notch signaling in EMT and suggested that inactivation of Notch signaling leads to the reversal of EMT to the MET phenotype with less invasive characteristics.

If activation of Notch-1 signaling contributes to gefitinib resistance, then inhibition of the Notch pathway could sensitize PC9/AB2 cells to gefitinib. Our results indicated that the viability of PC9/AB2 cells was synergistically decreased by Notch-1 siRNA in

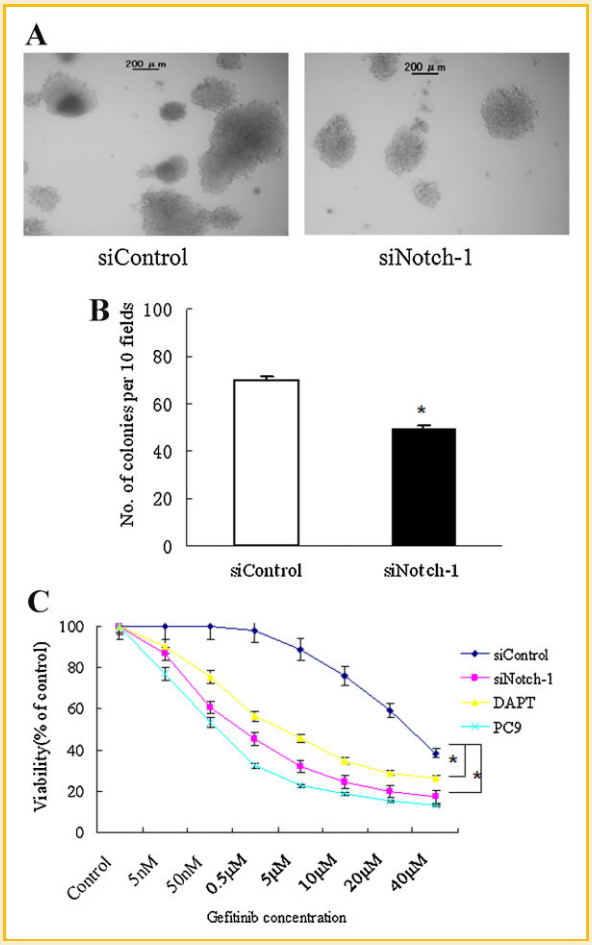

Fig. 8. Knockdown of Notch-1 using siRNA inhibits anchorage-independent cell growth and enhances chemosensitivity in PC9/AB2 cells. A: Anchorage-independent growth was studied using soft agar colony formation assays. Bar graphs represent colony formation in soft agar (scale bar, 200 μM). Two representative fields at ×100 magnifications for each cell line were captured for 14 days following seeding in soft agar. PC9/AB2 cells transfected with Notch-1 siRNA formed smaller colonies compared with control cells. B: The number of colonies that were 200 μM or larger per 10 fields at ×100 magnification was summarized in the right panel. Values are expressed as mean ± SD of at least three independent experiments. Asterisk (*) indicates significant differences compared to siControl control cells ($P < 0.05$). C: PC9/AB2 cells were transfected with siNotch-1 or siControl or treated with 10 μmol/L DAPT for 24 h, followed by treated with increasing concentrations of gefitinib for an additional 48 h. Afterwards, cell viability was measured with the CCK-8 kit and plotted as a percentage of the viability of untreated cells (control). Asterisk (*) indicates statistical significance when comparing PC9/AB2 cells transfected with control siRNA or Notch-1 siRNA; or when comparing PC9/AB2 parental cells with PC9/AB2 cells treated with DAPT ($P < 0.05$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

combination with gefitinib. Thus, down-regulation of Notch-1 might represent a novel mechanism to enhance the effect of EGFR-TKI and to delay resistance to EGFR-TKI in patients with NSCLC as EGFR-TKI has been correlated to NSCLC progression from the EMT phenotype [Uramoto et al., 2010].

In conclusion, our results provide strong molecular evidence demonstrating that gefitinib-acquired resistant lung cancer cells undergoing EMT partially through activation of Notch-1. Thus, down-regulation of Notch-1 signaling appears to be a novel strategy for the reversal of the EMT phenotype and increasing EGFR-TKI sensitivity to lung cancer treatment.

ACKNOWLEDGMENTS

We thank Dr. Chao-sheng He for his valuable suggestions of experiment design.

REFERENCES

Bienvenu F, Jirawatnotai S, Elias JE, Meyer CA, Mizeracka K, Marson A, Frampton GM, Cole MF, Odom DT, Odajima J, Geng Y, Zagozdzon A, Jecrois M, Young RA, Liu XS, Cepko CL, Gygi SP, Sicinski P. 2010. Transcriptional role of cyclin D1 in development revealed by a genetic–proteomic screen. Nature 463:374–378.

Casas E, Kim J, Bendesky A, Ohno-Machado L, Wolfe CJ, Yang J. 2011. Snail2 is an essential mediator of Twist1-induced epithelial mesenchymal transition and metastasis. Cancer Res 71:245–254.

Castella P, Sawai S, Nakao K, Wagner JA, Caudy M. 2000. HES-1 repression of differentiation and proliferation in PC12 cells: Role for the helix 3-helix 4 domain in transcription repression. Mol Cell Biol 20:6170–6183.

Cataldo VD, Gibbons DL, Perez-Soler R, Quintas-Cardama A. 2011. Treatment of non-small-cell lung cancer with erlotinib or gefitinib. N Engl J Med 364:947–955.

Daubriac J, Fleury-Feith J, Kheuang L, Galipon J, Saint-Albin A, Renier A, Giovannini M, Galateau-Sallé FM, Jaurand MC. 2009. Malignant pleural mesothelioma cells resist anoikis as quiescent pluricellular aggregates. Cell Death Differ 16:1146–1155.

Denlinger CE, Ikonomidis JS, Reed CE, Spinale FG. 2010. Epithelial to mesenchymal transition: The doorway to metastasis in human lung cancers. J Thorac Cardiovasc Surg 140:505–513.

Foubert E, De Craene B, Berx G. 2010. Key signalling nodes in mammary gland development and cancer. The Snail1–Twist1 conspiracy in malignant breast cancer progression. Breast Cancer Res 12:206–216.

Ju L, Zhou C, Li W, Yan L. 2010. Integrin beta1 over-expression associates with resistance to tyrosine kinase inhibitor gefitinib in non-small cell lung cancer. J Cell Biochem 111:1565–1574.

Koizumi F, Shimoyama T, Taguchi F, Saijo N, Nishio K. 2005. Establishment of a human non-small cell lung cancer cell line resistant to gefitinib. Int J Cancer 116:36–44.

Li J, Zhou BP. 2011. Activation of beta-catenin and Akt pathways by Twist are critical for the maintenance of EMT associated cancer stem cell-like characters. BMC Cancer 11:49–59.

Liu M, Casimiro MC, Wang C, Shirley LA, Jiao X, Katiyar S, Ju X, Li Z, Yu Z, Zhou J, Johnson M, Fortina P, Hyslop T, Windle JJ, Pestell RG. 2009. p21CIP1 attenuates Ras- and c-Myc-dependent breast tumor epithelial mesenchymal transition and cancer stem cell-like gene expression in vivo. Proc Natl Acad Sci USA 106:19035–19039.

Luna-Zurita L, Prados B, Grego-Bessa J, Luxan G, del Monte G, Benguria A, Adams RH, Perez-Pomares JM, de la Pompa JL. 2010. Integration of a Notch-dependent mesenchymal gene program and Bmp2-driven cell invasiveness regulates murine cardiac valve formation. J Clin Invest 120:3493–3507.

Munoz J, Zhou Y, Jarrett HW. 2010. LG4-5 domains of laminin-211 binds alpha-dystroglycan to allow myotube attachment and prevent anoikis. J Cell Physiol 222:111–119.

Nakamura T, Ohtsuka T, Sekiyama E, Cooper LJ, Kokubu H, Fullwood NJ, Barrandon Y, Kageyama R, Kinoshita S. 2008. Hes1 regulates corneal development and the function of corneal epithelial stem/progenitor cells. Stem Cells 26:1265–1274.

Nevins JR. 2011. Pathway-based classification of lung cancer: A strategy to guide therapeutic selection. Proc Am Thorac Soc 8:180–182.

Onitsuka T, Uramoto H, Nose N, Takenoyama M, Hanagiri T, Sugio K, Yasumoto K. 2010. Acquired resistance to gefitinib: The contribution of mechanisms other than the T790M, MET, and HGF status. Lung Cancer 68: 198–203.

Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M. 2004. EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. Science 304:1497–1500.

Pannuti A, Foreman K, Rizzo P, Osipo C, Golde T, Osborne B, Miele L. 2010. Targeting Notch to target cancer stem cells. Clin Cancer Res 16:3141–3152.

Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H, Aster JC, Krishna S, Metzger D, Chambon P, Miele L, Aguet M, Radtke F, Dotto GP. 2001. Notch signaling is a direct determinant of keratinocyte growth arrest entry into differentiation. EMBO J 20(13):3427–3436.

Saad S, Stanners SR, Yong R, Tang O, Pollock CA. 2010. Notch mediated epithelial to mesenchymal transformation is associated with increased expression of the Snail transcription factor. Int J Biochem Cell Biol 42: 1115–1122.

Thiery JP, Acloque H, Huang RY, Nieto MA. 2009. Epithelial–mesenchymal transitions in development and disease. Cell 139:871–890.

Uramoto H, Iwata T, Onitsuka T, Shimokawa H, Hanagiri T, Oyama T. 2010. Epithelial–mesenchymal transition in EGFR-TKI acquired resistant lung adenocarcinoma. Anticancer Res 30:2513–2517.

Uramoto H, Shimokawa H, Hanagiri T, Kuwano M, Ono M. 2011. Expression of selected gene for acquired drug resistance to EGFR-TKI in lung adenocarcinoma. Lung Cancer. 73:361–365.

Wang Z, Li Y, Kong D, Banerjee S, Ahmad A, Azmi AS, Ali S, Abbruzzese JL, Gallick GE, Sarkar FH. 2011. Acquisition of epithelial–mesenchymal transition phenotype of gemcitabine-resistant pancreatic cancer cells is linked with activation of the notch signaling pathway. Cancer Res 69:2400–2407.

Yeh TS, Lin YM, Hsieh RH, Tseng MJ. 2003. Association of transcription factor YY1 with the high molecular weight Notch complex suppresses the transactivation activity of Notch. J Biol Chem 278:41963–41969.

Yilmaz M, Christofori G. 2009. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev 28:15–33.

Zhang W, Jiang B, Guo Z, Sardet C, Zou B, Lam CS, Li J, He M, Lan HY, Pang R, Hung IF, Tan VP, Wang J, Wong BC. 2010. Four-and-a-half LIM protein 2 promotes invasive potential and epithelial–mesenchymal transition in colon cancer. Carcinogenesis 31:1220–1229.