Knockdown of *ZEB1*, a master epithelial-to-mesenchymal transition (EMT) gene, suppresses anchorage-independent cell growth of lung cancer cells

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Abstract

We found that among four master epithelial-to-mesenchymal transition (EMT)-inducing genes (*ZEB1*, *SIP1*, *Snail*, and *Slug*) *ZEB1* expression was most significantly correlated with the mesenchymal phenotype (high *Vimentin* and low *E-cadherin* expression) in non-small cell lung cancer (NSCLC) cell lines and tumors. Furthermore, *ZEB1* knockdown with RNA interference in three NSCLC cell lines with high *ZEB1* expression suppressed to varying degrees mass culture growth and liquid colony formation but in all cases dramatically suppressed soft agar colony formation. In addition, *ZEB1* knockdown induced apoptosis in one of the three lines, indicating that the growth inhibitory effects of *ZEB1* knockdown occurs in part through the activation of the apoptosis pathway. These results suggest that inhibiting *ZEB1* function may be an attractive target for NSCLC therapeutic development.

Keywords

Lung cancer; Epidermal growth factor receptor; Anchorage-independent growth; EMT; MicroRNA; RNA interference

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5. Conflict of interest
We have no conflict of interest to be disclosed.

1. Introduction

Epithelial-to-mesenchymal transition (EMT) is an embryonic developmental program involving changes in cell morphology and expression of EMT-associated genes [1,2]. EMT also occurs during the progression of several types of human cancer and confers motility and invasiveness on cancer cells, leading them to acquire ability to metastasize to distant sites. Genetics of early development discovered a number of EMT-inducing genes encoding transcription factors capable of inducing EMT when ectopically expressed in epithelial cells. Several EMT-inducing genes that have essential roles in EMT are called master EMT genes, including *Twist*, *ZEB1*, *SIP1*, *Snail*, *Slug*, and Goosecoid [1,2]. These genes function as transcriptional repressors of the cell–cell adhesion glycoprotein, E-cadherin whose functional loss is one of the hallmarks of EMT [3]. Among these master EMT genes, *Snail* was shown to repress E-cadherin and to induce EMT in cancer cells [4,5], while *Twist* was demonstrated to promote breast cancer metastasis [6]. Increased expression of *Twist* and *Snail* have been shown in hepatocellular, breast, colorectal, and gastric cancers, often correlating with poor prognosis [7–10].

Lung cancer is the leading cause of cancer deaths, killing over 1 million people every year worldwide [11]. It develops through a multi-step process involving accumulation of multiple genetic and epigenetic changes that confer growth advantages on normal lung epithelial cells, leading them to transform to clinically evident lung cancer cells [12,13]. Analyzing a large number of lung tumor specimens, Prudkin et al. showed that the majority of primary lung cancers and even premalignant lesions have the mesenchymal phenotype as characterized by down-regulation of E-cadherin and up-regulation of Vimentin [14]. Although several master EMT genes have been shown to directly contribute to tumor progression in breast, colon, and pancreatic cancers, very little is known about functional roles of master EMT genes in lung cancer progression.

In addition to *Snail* and *Twist*, recently *ZEB1* has emerged as a key player in cancer progression. *ZEB1* promotes tumor metastasis in colon and breast cancer [15], is associated with resistance to conventional chemotherapy in pancreatic cancer [16,17], and potentially has a predominant role in inducing EMT in NSCLC. First, among several transcription factors including ZEB1, Snail, and β-catenin, ZEB1 protein expression showed the most significant inverse correlation with E-cadherin in NSCLC and mesothelioma cell lines [18]. Second, prostaglandin E2 was shown to exert its ability to suppress E-cadherin through inducing *ZEB1* and *Snail* in lung cancer cell lines [19]. Third, *ZEB1* has been shown to suppress the Semaphorin 3F tumor suppressor gene in lung cancer cells [20]. These suggest relevant roles of *ZEB1* as an EMT-inducer as well as an oncogene in lung cancer.

Thus, we performed this study aiming to evaluate the association between *ZEB1* expression and the mesenchymal phenotype in lung cancer, and to test the effects of *ZEB1* knockdown with RNA interference on the growth of lung cancer cells. We found that *ZEB1* expression significantly correlates with increased *Vimentin* and decreased *E-cadherin* expression in lung cancer, while knockdown of *ZEB1* resulted in dramatic growth inhibition in lung cancer cell lines. These results suggest that *ZEB1* is a promising therapeutic target for lung cancer.

2. Materials and methods

2.1. Cell lines and primary tumor tissues

NSCLC cell lines used in this study were purchased from American Type Culture Collection or obtained from the Hamon Center collection (University of Texas Southwestern Medical Center). These cells include PC9, A549, NCI-H157, NCI-H460, NCI-H820, NCI-H838,

NCI-H1155, NCI-H1299, NCI-H1666, NCI-H1650, NCI-H1975, NCI-H3255, HCC44, HCC827, HCC2279, HCC2935, HCC4006, and HCC4011 (cells with mutation in epidermal growth factor receptor (EGFR) gene are underlined) [21]. A mesothelioma cell line, ACC-MESO-1, which was used as positive control for western blot of cleaved caspase-3, was established by ourselves [22]. Cells were cultured with RPMI 1640 (Sigma–Aldrich Corp, MO, USA) supplemented with 10% fetal bovine serum. Surgically resected 32 primary tumor specimens (19 adenocarcinomas and 13 squamous cell carcinomas) were obtained from patients at the Nagoya University Hospital, Nagoya First Japan Red Cross Hospital, Nagoya Second Japan Red Cross Hospital, Kasugai Municipal Hospital and Chukyo Hospital in Nagoya, Japan. Before tissue samples were collected ethical approval of the each institute and fully informed written consents from all patients were obtained. We previously analyzed *EGFR* mutation status of these samples and used the data of the analysis for the present study [23].

2.2. RNA isolation and quantitative real-time PCR analysis

For mRNA analysis, 5 μg of total RNA isolated using Trizol (Invitrogen Corp., CA, USA) were reverse transcribed with Super script III First-Strand Synthesis System using Random primer system (Invitrogen Corp.). Quantitative real-time PCR (qRT-PCR) analysis of *E-cadherin*, *Vimentin*, *ZEB1*, *SIP1 Snail*, and *Slug*, was performed as described previously using the standard Taqman assay-on-demand PCR protocol in a reaction volume of 20 μL, including 50 ng cDNA [24]. We used the comparative C_t method to compute relative expression values. For microRNA analysis, 10 ng of total RNA isolated using *mir*Vana miRNA Isolation Kit (Applied Biosystems, CA, USA) were reverse transcribed with TaqMan MicroRNA Reverse Transcription Kit using a primer set specific for each of microRNAs (miR-200a, miR-200b, miR-200c, and miR-205) studied (Applied Biosystems). qRT-PCR analysis of microRNA was done as described above. We used *GAPDH* (Applied Biosystems assay-on-demand) for mRNA analysis and U6 small nuclear (sn) RNA for microRNA analysis as internal controls.

2.3. Western blot analysis

Western blot analysis was done as described previously using whole cell lysates [24]. Primary antibodies used were mouse monoclonal anti-E-cadherin, anti-Vimentin (BD Bioscience, NJ, USA), goat polyclonal anti-ZEB1 (Santa Cruz biotech., CA, USA), and rabbit polyclonal anti-cleaved caspase-3 (Cell Signaling Tech., MA, USA). Actin protein levels were used as a control for adequacy of equal protein loading. Anti-rabbit, anti-mouse (GE healthcare, Buckinghamshire, England), or anti-goat antibody (R&D Systems, MN, USA) was used at 1:2000 dilution as a secondary antibody.

2.4. Transfection of short interfering RNA

The 4.5×10^5 of H1299 and H157 or 9.0×10^5 of H460 cells were plated in 6-well plates. Next day, cells were transiently transfected with either 10 nM predesigned short interfering RNA (siRNA) (Stealth Select RNAi) targeting *ZEB1* or control siRNA purchased from Invitrogen using Lipofectamine RNAiMAX (Invitrogen Corp.) according to the manufacturer's protocol. After 48 h, the transfected cells were harvested for further analyses or plated for cell growth assays.

2.5. Cell growth assays

Colorimetric proliferation assay was performed using WST-1 assay kit (Roche, Basel, Switzerland) according to manufacturer's instruction. Liquid and soft agar colony formation assays were done as described previously [24].

2.6. Cell cycle analysis

Cells were harvested 48 h after the transfection of siRNA oligos. Cells were, fixed, treated with RNase A, stained with propidium iodide using BD Cycletest Plus Reagent Kit (BD Bioscience) according to the instructions of the manufacture, and analyzed by flow cytometry for DNA synthesis and cell cycle status [FACSCalibur instrument, (Becton Dickinson) with BD CellQuest™Pro Ver.5.2.1 (BD Bioscience)].

2.7. Senescence associated β-galactosidase staining

Cells were stained with β-galactosidase using Senescence β-Galactosidase Staining Kit (Cell Signaling Tech.), and cells stained blue were counted under a microscope (200× total magnification).

2.8. Statistics

SPSS ver.17 software was used for all statistic analyses in this study. Spearman's correlation coefficients with associated *P* values were calculated between mRNA expression of *Vimentin*, *E-cadherin*, and four EMT-inducing genes, microRNA expression of the four microRNAs, and the ratio of *Vimentin* to *E-cadherin* expression (RVE). Mann–Whitney *U* test was used for analyzing difference between two groups.

3. Results

3.1. Lung cancer cell lines can be divided into epithelial and mesenchymal phenotypes based on the expression status of E-cadherin and Vimentin

We first examined whether lung cancer cell lines can be classified into mesenchymal and epithelial phenotypes based on expression status of *E-cadherin* and *Vimentin*, which are markers for epithelial and mesenchymal phenotypes, respectively. qRT-PCR analysis of *E-cadherin* and *Vimentin* in 18 non-small cell lung cancer cell (NSCLC) lines including 10 with mutations in the epidermal growth factor receptor (*EGFR*) gene showed that most predominantly expressed either *E-cadherin* or *Vimentin* (Fig. 1A). We classified NSCLCs as either "epithelial" (high *E-cadherin*/low *Vimentin*) or "mesenchymal" (high Vimentin/low E-cadherin) according to the expression status of *E-cadherin* and *Vimentin*. We quantified the ratio of *Vimentin* to *E-cadherin* expression (RVE) as an index that represents the degree of mesenchymal phenotype, with NSCLCs showing RVE ≥ 1.0 classified as mesenchymal phenotype (*n* = 9 NSCLCs) and those with RVE < 1.0 as epithelial phenotype (*n* = 9 NSCLCs). Notably, RVEs of *EGFR* wild-type NSCLCs were significantly higher than those of *EGFR* mutant NSCLCs (Fig. 1B) (Median; 80.0 vs. 0.06, *P* = 0.008, Mann–Whitney *U* test). All but one *EGFR* wild-type NSCLC lines were "mesenchymal" while 8 of 10 *EGFR* mutant NSCLCs were "epithelial", suggesting that *EGFR* mutation is associated with epithelial characteristics (Fig. 1B).

3.2. Among four master EMT genes only ZEB1 expression was significantly correlated with both Vimentin and E-cadherin expression in lung cancer cell lines

To identify master EMT genes whose expressions are significantly associated with the mesenchymal phenotype in lung cancer cell lines, we analyzed the expression levels of four master EMT genes (*ZEB1*, *SIP1*, *Snail*, and *Slug*) (Fig. 1A, Table 1). *ZEB1* expression was inversely correlated with *E-cadherin* expression (Spearman's correlation coefficient = −0.82, *P* < 0.001), and positively correlated with *Vimentin* expression (Spearman's correlation coefficient = 0.80, *P* < 0.001) (Table 1), resulting in highly significant association between *ZEB1* expression and RVE (Spearman's correlation coefficient = 0.88, *P* < 0.001) (Table 1). By contrast, *SIP1*, *Snail*, and *Slug* expression were not correlated with

E-cadherin expression, *Vimentin* expression, or RVE (Table 1). These results suggested that *ZEB1* may induce EMT in lung cancer.

3.3. The expression levels of four miroRNAs known as repressors of ZEB1 were negatively correlated with ZEB1 expression in NSCLCs

Recently, several groups have shown that *ZEB1* expression is down-regulated by three members of microRNA-200 family (miR-200a, miR-200b, miR-200c) and miR-205 in different types of cancer cells [25–28]. To confirm these findings in a panel of lung cancer cell lines, we performed qRT-PCR of the four microRNAs in 12 NSCLC cell lines and correlated their expression with *ZEB1* expression (Fig. 1C). We found that the expression levels of miR-200c and miR-205 were negatively correlated with *ZEB1* expression (Spearman's correlation coefficients: −0.71, $P = 0.01$ for miR-200c, −0.79, $P = 0.002$ for miR-205), while miR-200a and miR-200b were not correlated with *ZEB1* expression. These results suggest that miR-200c and miR-205 may have a major role in regulating *ZEB1* expression in lung cancer (Fig. 1C).

3.4. ZEB1 and Snail expression were significantly correlated with RVE in primary lung tumor tissues

Next, we analyzed the expression of *E-cadherin*, *Vimentin*, and the four master EMT genes in 32 NSCLC tumor specimens and found that *ZEB1* expression was highly correlated with *Vimentin* expression (Spearman's correlation coefficient = 0.92, $P < 0.001$) (Table 2). *ZEB1* (Spearman's correlation coefficient = 0.51, $P = 0.003$), *and Snail* (Spearman's correlation coefficient = 0.45, $P = 0.01$) expression were significantly correlated with RVE. However, we note that overall in the tumor specimens in contrast to the NSCLC lines, *E-cadherin* and *Vimentin* expression were positively correlated (Spearman's correlation coefficient = 0.63, $P < 0.001$) (Table 2). This may be because the primary tumor specimens were macrodissected and thus included transcripts from both tumor and non-tumorous lung tissue while the NSCLC lines only reflected tumor expression. Probably because of this reason, unlike in the NSCLC lines, *EGFR* mutation status was not correlated with epithelial characteristics (high *E-cadherin* and low *Vimentin*) in the primary tumors (data not shown).

3.5. Knockdown of ZEB1 induced significant suppression of anchorage-independent cell growth in lung cancer cell lines

The results presented above suggest that *ZEB1* plays a dominant role in maintaining the mesenchymal phenotype in NSCLCs. To test the therapeutic potential of *ZEB1* we performed RNA interference (RNAi)-mediated gene silencing against *ZEB1*. To minimize the possibility of "off target effect", we used low dose (10 nM) Stealth Select RNAi (Invitrogen), which includes three short interfering RNA (siRNA) oligos with non-overlapping sequences targeting *ZEB1*. We studied three NSCLC cell lines, H1299, H460, and H157, all of which express high levels of *ZEB1* mRNA (Fig. 1A). These cell lines were transiently transfected with each of the three *ZEB1* siRNAs or control oligos and harvested for analyses 48 h after transfection. Western blot of ZEB1 showed that with all three oligos clear suppression of ZEB1 protein was obtained in all cell lines (Fig. 2A). Western blot of E-cadherin, which is a direct negative transcription target of *ZEB1*, showed reexpression of E-cadherin protein in H460 but not in H1299 or H157 after the transfection (Fig. 2A). This was likely to reflect the difference in basal expression of E-cadherin protein between the cells; H460 but not H1299 or H157 expressed detectable levels of E-cadherin protein (Fig. 2A). However, we also considered the possibility that *ZEB1* knockdown did not upregulate *E-cadherin* mRNA in H1299 or H157 and thus performed qRT-PCR analysis of *E-cadherin*. The analysis revealed that after the *ZEB1* knockdown transfection *E-cadherin* mRNA significantly increased in H157 and H460 but remained unchanged in H1299, indicating that in H1299 *E-cadherin* mRNA was not upregulated by *ZEB1* knockdown (Fig. 2B). Small or

no changes in Vimentin protein expression were seen after the transfection of *ZEB1* knockdown oligos (Fig. 2A). Finally, we did not see significant morphologic changes suggestive of the cells undergoing EMT (data not shown).

We found *ZEB1* knockdown to have modest effects suppressing mass culture and liquid colony formation growth but much greater effects suppressing anchorage-independent growth in soft agar (Fig. 2C–E). This result indicates that the ability of these cells to grow in soft agar is highly dependent on *ZEB1* expression.

3.6. Growth inhibitory effect of ZEB1 knockdown in lung cancer was caused in part by apoptosis but not by induction of senescence

The NSCLC cell lines varied in the induction of apoptosis following *ZEB1* knockdown with H460 cells showing the largest amount of *ZEB1* knockdown-induced apoptosis (Fig. 3A and B). One study has showed that mouse embryonic fibroblasts derived from Zeb1 −/− mice underwent premature senescence, suggesting that *ZEB1* may function as inhibitor of senescence [29]. Nevertheless, we did not see any increase in the number of cells exhibiting morphologic changes or β-galactosidase staining suggestive of senescence, in any of cells transfected with *ZEB1* knockdown oligos (data not shown).

4. Discussion

In the present study, we have shown that *ZEB1* expression was correlated with the mesenchymal phenotype in NSCLC, and that its depletion with RNA interference suppressed anchorage-independent growth, thus providing a rationale for developing therapeutics targeting *ZEB1* function in lung cancer. Consistent with reports in other cancers, we also found that miR-200c and miR-205 expression were inversely correlated with *ZEB1* expression in NSCLCs, suggesting potential use as therapy targeting *ZEB1*.

Consistent with the literature [30], we found that *EGFR* mutations were associated with epithelial characteristics (E-cadherin expression) in NSCLCs. The literature study showed that *EGFR* mutant genotype is an independent predictor for the epithelial phenotype in logistic regression analysis, excluding the possibility that observed high frequency of the epithelial phenotype in *EGFR* mutants was due to other factors that may be associated with or may cause epithelial phenotype. Such factors include early stage disease and non- or low smoking history. The finding that *EGFR* mutant cells frequently have an epithelial phenotype seems to be unexpected because signaling pathways activated by *EGFR* including RAS, AKT, and SRC pathways can induce EMT [31]. It is possible that *EGFR* mutant cells are resistant to EMT-inducing signals. Alternatively, it is also possible that mutant cells specifically express genes that retain cells to the epithelial state. It will be important to further elucidate the molecular mechanisms leading to epithelial phenotype in *EGFR* mutant cells because molecules involved in such mechanisms could serve as therapeutic targets.

We found that in NSCLC cell lines *ZEB1* expression (in contrast to othermaster EMT genes) was significantly correlated with both *Vimentin* and *E-cadherin* expression. In addition, we saw that *ZEB1* expression was very strongly correlated with *Vimentin* expression in primary tumors. Two previous studies also reported that *ZEB1* expression was most significantly correlated with E-cadherin expression in lung cancer among several EMT-inducing genes [18,32]. Although *ZEB1* has been shown to directly repress transcription of *E-cadherin* in several different types of cells [33,34], it remains unknown whether *ZEB1* up-regulates Vimentin expression. In our *ZEB1* knockdown experiments, we did not see a significant down-regulation of Vimentin in any of the three lines studied, suggesting that *ZEB1* does not directly up-regulate Vimentin expression. However, the observed strong correlation between *ZEB1* and *Vimentin* expression in both NSCLC cell lines and tumors suggests that *ZEB1*

may indirectly up-regulate *Vimentin* possibly through some of its downstream targets. Collectively, our results, along with those of others, suggest a dominant role of *ZEB1* in maintaining the mesenchymal phenotype of lung cancer.

ZEB1 knockdown induced *E-cadherin* mRNA expression in H157 and H460 but not in H1299. There is one possible explanation for this. In H1299, the promoter region of *E-cadherin* is known to be heavily methylated, causing its silenced expression [35]. Thus, it is possible that transient knockdown of *ZEB1* was unable to overcome methylation- mediated gene silencing of *E-cadherin* in H1299, resulting in the unchanged *E-cadherin* mRNA. Long-term *ZEB1* knockdown could reverse methylation of *E-cadherin*, and thus it wound be interesting to see the effects of stable *ZEB1* knockdown on methylation status of *E-cadherin* in H1299.

ZEB1 knockdown inhibited NSCLC growth most dramatically noted in soft agar colony formation assay. Since the ability of transformed cells to grow under anchorage-independent condition is the most reliable predictor for tumorigenicity and metastatic potential, this strong growth inhibitory effect of *ZEB1* knockdown in anchorage-independent condition suggests that *ZEB1* expression contributes to maintaining aggressive phenotype of lung cancer cells.

Studies have shown that *Twist*, *Snail* and *SIP1* show antiapoptotic effects when ectopically expressed [36–38]. Nevertheless, to our knowledge, only one study has demonstrated anti-apoptotic effect of a master EMT gene in a gene-silencing experiment; the study demonstrated that *Twist* knockdown induced apoptosis in human neuroblastoma cells [39]. We also observed that *ZEB1* knockdown induced apoptosis in H460. These results including ours demonstrate that in some cancer cells survival signals are dependent on expression of master EMT genes such as *Twist* and *ZEB1*. Cancer cells are thought to acquire the ability to evade apoptosis in early stage of progression, and thus these finding suggest that master EMT genes may play an important role not only in late stage but also in early stage of carcinogenesis.

In conclusion, we have shown that *ZEB1* expression is very well correlated with the mesenchymal phenotype of NSCLC, and that its removal induces significant growth inhibition in NSCLC partially through induction of apoptosis. These results suggest that *ZEB1* is a promising therapeutic target for lung cancer.

Acknowledgments

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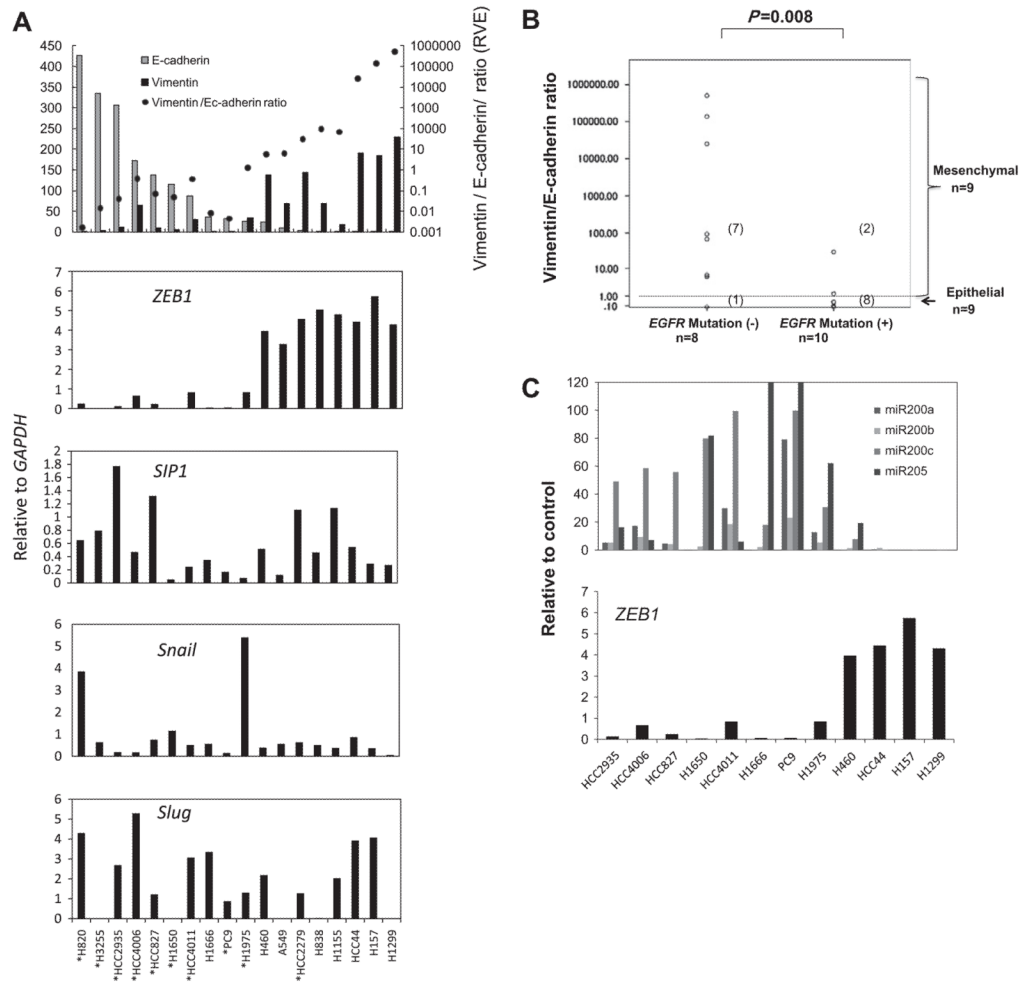
Fig. 1.
ZEB1 expression significantly correlates with *E-cadherin*, *Vimentin* and two microRNAs, miR-200c and miR-205, known *ZEB1* repressor in lung cancer cell lines. (A) Quantitative real-time PCR (qRT-PCR) analysis of *E-cadherin*, *Vimentin* and four master EMT genes, *ZEB1*, *SIP1*, *Snail*, and *Slug* in 18 lung cancer cell lines. The cell lines are aligned by expression levels of *E-cadherin* from high (left) to low (right). The results are average of two independent PCR experiments done in duplicated reactions. Statistic values of correlations between these genes are shown in Table 1. *Cells with mutation in the epidermal growth factor receptor (*EGFR*) gene. (B) The association between the ratio of *Vimentin* to *E-cadherin* (RVE) and mutation status of *EGFR* in lung cancer cell lines. (C) qRT-PCR analysis of four microRNAs known as repressors of *ZEB1*. The expression levels of miR-200c and miR-205 were significantly negatively associated with *ZEB1* expression (Spearman's correlation coefficients: −0.71, *P* = 0.01 for miR-200c and −0.79, *P* = 0.002 for miR-205).

A

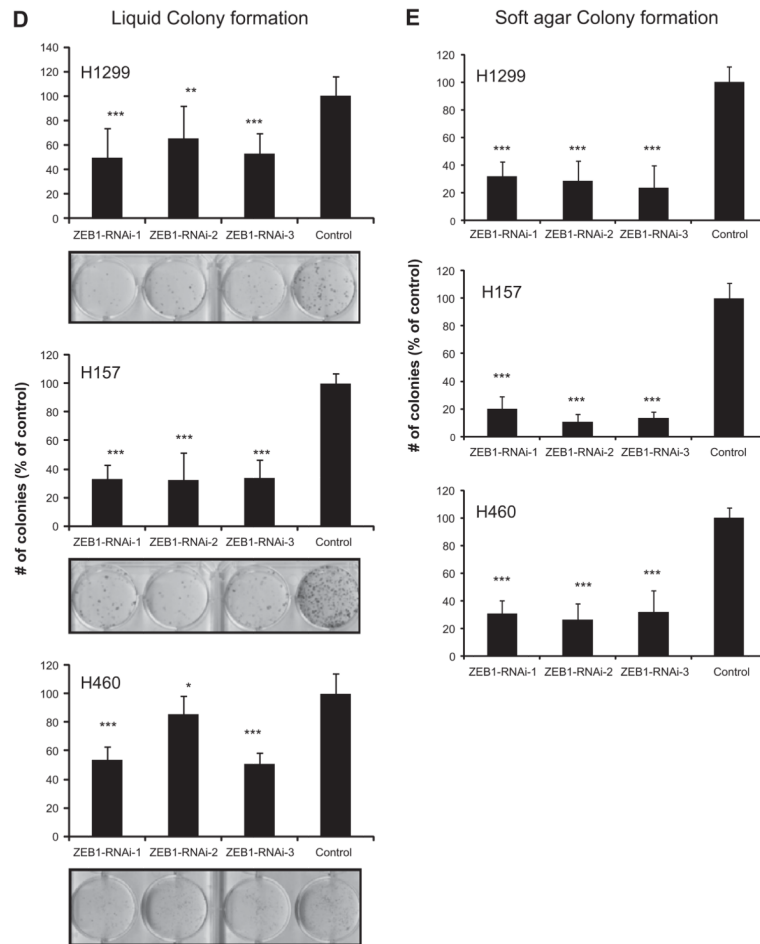**B** *E-cadherin* mRNA

C WST-1 assay
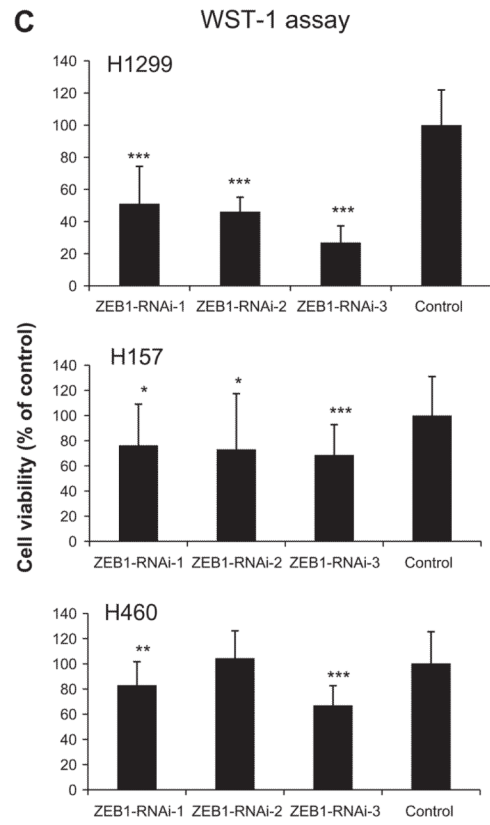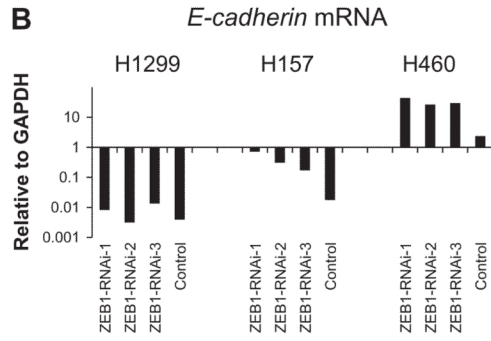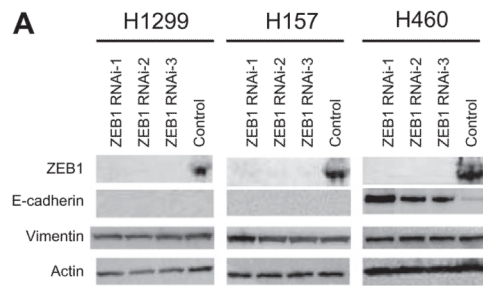
Fig. 2.
RNAi-mediated knockdown of *ZEB1* suppresses cell proliferation, liquid colony formation (anchorage-dependent condition), and soft agar colony formation (anchorage-independent condition) in lung cancer cells. (A) Western blot of ZEB1, E-cadherin, and Vimentin for lung cancer cell lines transfected with *ZEB1* knockdown or control oligos. Actin was used as a loading control. The result is a representative result of two independent experiments. (B) qRT-PCR analysis of *E-cadherin* for lung cancer cell lines transfected with *ZEB1* knockdown or control oligos. The result is an average of three independent experiments done in duplicates, (C) Cell proliferation (WST-1) assay of lung cancer cell lines transfected with *ZEB1* knockdown or control oligos. Cells were transfected with three different RNAi oligos targeting *ZEB1* or control oligos. Forty-eight hours after the transfection cells were counted and 1000 cells were plated in 96-well plates. Absorbance values were determined 96 h after the transfection. Results are from three independent experiments in eight replicates and shown as mean ± SD. Values of cells transfected with control oligos are set as 100%. (D) Liquid colony formation assay for lung cancer cell lines transfected with *ZEB1* knockdown or control oligos. Forty-eight hours after the transfection cells were counted and 500 or 1000 cells were plated in 6-well plates in triplicates, cultured for two weeks, and stained with methylene blue. Colonies >2 mm were counted. Results are from three independent experiments and shown as mean ± SD. Colony numbers of cells transfected with control oligos are set as 100%. (E) Soft agar colony formation assay for lung cancer cell lines transfected with *ZEB1* knockdown or control oligos. Forty-eight hours after the transfection cells were counted and 1000 cells were suspended in 0.37% SeaKem GTG

Agarose (Lonza, Rockland, ME, USA) in triplicate 12-well plates. About 2 weeks later, colonies (>50 cells) were counted. Results are from three independent experiments and shown as mean ± SD. Colony numbers of cells transfected with control oligos are set as 100%. In Fig. 2C–E, *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ (Mann–Whitney U test), respectively.
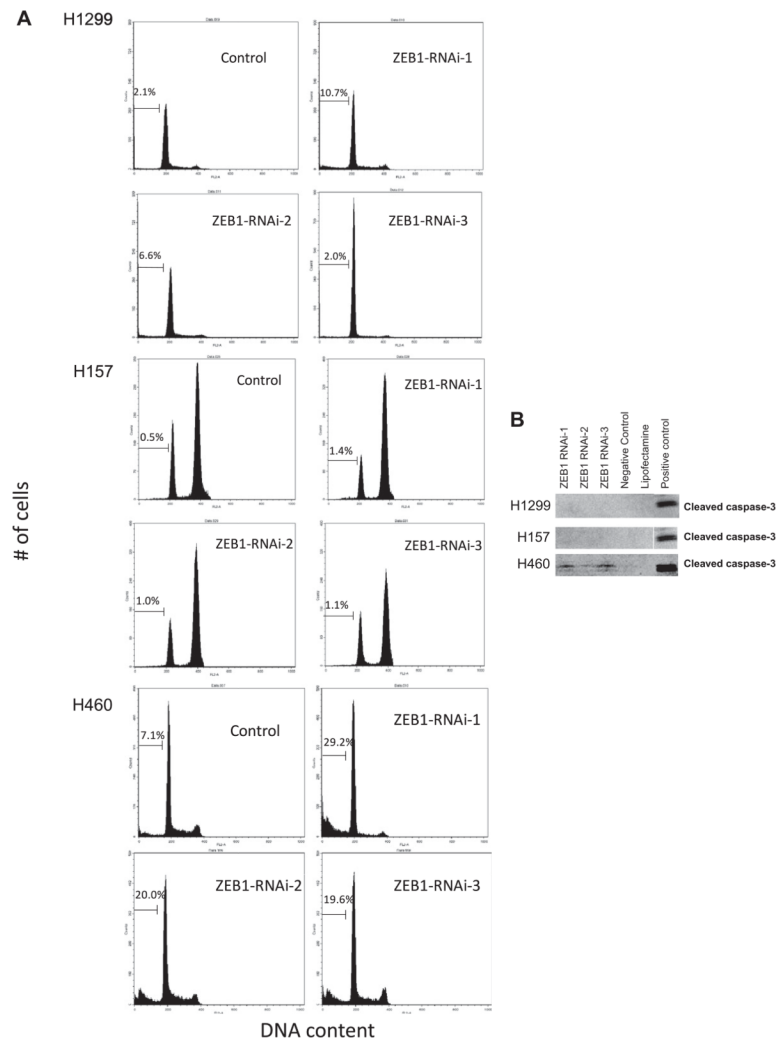
Fig. 3.
ZEB1 knockdown induces apoptosis in H460. (A) Flow cytometry analysis for lung cancer cell lines transfected with *ZEB1* knockdown or control oligos. Forty-eight hours after the transfection cells were stained with propidium iodine and 20,000 cells were analyzed for cell cycle profiling. Representative results in three independent experiments are shown. (B) Western blot of cleaved caspase-3 for lung cancer cell lines transfected with *ZEB1* knockdown or control oligos. ACC-MESO-1 mesothelioma cell line treated with cisplatin was used as a positive control.

Table 1

Correlations between mRNA expression of master EMT genes, E-cadherin, and Vimentin in 18 non-small lung cancer cell lines.

	E-cadherin	Vimentin	RVE	ZEB1	SIP1	Snail
E-cadherin Vimentin	−0.74					
	<0.001					
RVE	−0.88	0.92				
	<0.001	<0.001				
ZEB1	−0.82	0.80	0.88			
	<0.001	<0.001	<0.001			
SIP1	0.22	−0.05	−0.06	−0.06		
	0.38	0.85	0.81	0.80		
Snail	0.30	−0.22	−0.26	−0.23	−0.03	
	0.23	0.38	0.30	0.36	0.92	
Slug	0.09	0.07	−0.04	0.20	0.24	−0.06
	0.72	0.79	0.88	0.46	0.34	0.82

Spearman's correlation coefficients (upper row) and statistic values (lower row) are shown. Statistically significant correlations ($P < 0.01$) are in bold. RVE; the ratio of *Vimentin* to *E-cadherin*.

Table 2

Correlations between mRNA expression of master EMT genes, E-cadherin, and Vimentin in 32 non-small lung cancer tumor tissues.

	E-cadherin	Vimentin	RVE	ZEB1	SIP1	Snail
E-cadherin Vimentin	**0.63** **<0.001**					
RVE	−0.26 0.15	**0.53** **0.002**				
ZEB1	**0.57** **<0.001**	**0.92** **<0.001**	**0.51** **0.003**			
SIP1	**0.81** **<0.001**	**0.73** **<0.001**	0.09 0.61	**0.64** **<0.001**		
Snail	0.32 0.07	**0.71** **<0.001**	0.45 0.01	**0.69** **<0.001**	**0.49** **0.004**	
Slug	0.29 0.11	0.39 0.03	0.31 0.08	0.41 0.02	0.43 0.01	0.26 0.15

Spearman's correlation coefficients (upper row) and statistic values (lower row) are shown. Statistically significant correlations ($P < 0.01$) are in bold. RVE; the ratio of *Vimentin* to *E-cadherin*.