Silencing of *GRHL2* induces epithelial-to-mesenchymal transition in lung cancer cell lines with different effects on proliferation and clonogenic growth

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Abstract. Grainyhead-like 2 (GRHL2) is a transcription factor that suppresses epithelial-to-mesenchymal transition (EMT). It has been previously shown that GRHL2 can confer both oncogenic and tumor-suppressive roles in human cancers, including breast, pancreatic and colorectal cancers. However, its role in lung cancer remains elusive. In the present study, a meta-analysis of multiple gene expression datasets with clinical data revealed that GRHL2 expression was increased in lung cancer compared with that in the normal tissues. Copy number analysis of *GRHL2*, performed using datasets of whole exome sequencing involving 151 lung cancer cell lines, revealed frequent amplifications, suggesting that the increased *GRHL2* expression may have resulted from gene amplification. A survival meta-analysis of *GRHL2* using The Cancer Genome Atlas (TCGA) dataset showed no association of *GRHL2* expression with overall survival. *GRHL2* expression was found to be associated with EMT status in lung cancer in TCGA dataset and lung cancer cell lines. *GRHL2* knockdown induced partial EMT in the *hTERT*/*Cdk4*-immortalized normal lung epithelial cell line HBEC4KT without affecting proliferation measured by CCK-8 assays. In addition, *GRHL2* silencing caused three lung cancer cell lines, H1975, H2009 and H441, to undergo partial EMT. However, the proliferative effects differed significantly. *GRHL2* silencing promoted proliferation but not colony formation in H1975 cells whilst suppressing colony formation without affecting proliferation in H2009 cells, but it did not affect proliferation in H441 cells. These results suggest cell type-dependent effects of *GRHL2* knockdown. Downstream, *GRHL2* silencing enhanced the phosphorylation of AKT and ERK, assessed by western blotting with phospho-specific antibodies, in HBEC4KT, H1975 and H2009 cell lines but not in the H441 cell line. By contrast, transient *GRHL2* overexpression did not affect A549 cell proliferation, which lack detectable endogenous expression of the GRHL2 protein. However, GRHL2 overexpression did suppress E-cadherin expression in A549 cells. These results suggested that *GRHL2* does not only function as a tumor suppressor of EMT but can also behave as an oncogene depending on the lung cancer cell-type context.

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Introduction

During epithelial-mesenchymal transition (EMT), which was first discovered in embryonic developmental studies, epithelial cells undergo notable changes in morphology, as characterized by polarized and tightly connected to adjacent cells to being less polarized and loosely connected, with spindle-like morphology and acquire functional properties of mesenchymal cells, such as a motile and invasive phenotype (1-3). Cancer cells acquires invasiveness and metastatic potential, by exploiting the EMT machinery that endows the cells with a motile and invasive phenotype (4).

EMT is regulated by several EMT-inducing transcription factors that serve as master regulators of EMT (3,4). By contrast, a number of EMT-repressing transcription factors have also been discovered (3). Among them, *Grainyhead-like 2* (*GRHL2*) encodes a transcription factor

that has been reported to regulate wound healing, tubulogenesis and cancer (5,6). However, *GRHL2* can exert both tumor-promoting and -suppressive functions depending on the human cancer involved (7-21). In pancreatic cancer, *GRHL2* functions as a tumor-promoting gene by conferring metastatic potential to cancer cells, by retaining epithelial but acquiring stem cell properties (11). By contrast, in breast cancer *GRHL2* inhibits anchorage-independent growth, where the loss of GRHL2 expression has been found to be associated with higher tumor stages (14). This suggests the tumor-suppressive role of GRHL2 in breast cancer (14). In addition, another study has previously reported tumor-suppressive roles of *GRHL2* in breast cancer, by demonstrating that suppression of EMT by *GRHL2* increased sensitivity to anoikis (10).

To the best of our knowledge, few reports have investigated the roles of *GRHL2* in lung cancer (12). *GRHL2* is overexpressed in lung cancer compared with that in normal cells, where it was associated with inferior prognosis. *GRHL2* silencing was reported to suppress proliferation and colony formation whilst enhancing migration and invasion. These data are difficult to interpret, because the results of proliferation and colony formation, together with increased *GRHL2* expression, suggested that GRHL2 serves oncogenic roles, but those from migration and invasion assays suggested a tumor-suppressive role. Furthermore, *GRHL2* has been demonstrated to exert oncogenic roles by stabilizing a 'hybrid epithelial/mesenchymal phenotype' in H1975 lung cancer cells (22). In that particular study, this 'hybrid epithelial/mesenchymal phenotype' was considered to be a promoter of cancer metastasis. These findings are contradictory to some extent and suggest a highly complex role of *GRHL2* in lung cancer. This justifies further investigations into the roles of *GRHL2* in lung cancer pathophysiology.

Therefore, in the present study, the role of *GRHL2* in lung cancer was investigated using the online bioinformatic tool, Lung Cancer Explorer (LCE) and preclinical models, including *hTERT*/*Cdk4*-immortalized normal lung epithelial cells and lung cancer cell lines (23-26).

Materials and methods

Cell culture. All 10 of lung cancer cell lines (HCC827, HCC4006, HCC4017, A549, H460, H441, H661, H1792, H1975 and H2009) used in the present study and the *hTERT*/*Cdk4*-immortalized normal human bronchial epithelial cell lines HBEC3KT and HBEC4KT were purchased from American Type Culture Collection and were derived from the Hamon Center Collection University of Texas Southwestern Medical Center (Dallas, USA) (27,28). The lung cancer cell lines were cultured in RPMI-1640 (cat. no. 189-02025; FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% FBS (cat. no 35-079-CV; Corning, Inc.). HBEC3KT and HBEC4KT cells were cultured in keratinocyte-serum free medium (cat. no. 10724-011; Thermo Fisher Scientific, Inc.) supplemented with 5 ng/ml epidermal growth factor (cat. no. PHG0314; Thermo Fisher Scientific, Inc.) and 50 ng/ml bovine pituitary extract (cat. no. 13028-014; Thermo Fisher Scientific, Inc.). All cells were cultured at 37˚C in a humidified 5% CO_2 incubator. The provenance of H1975, H2009 and H441 was confirmed by DNA fingerprinting short tandem repeat analysis. Negativity for mycoplasma contamination in the cultured cell lines was confirmed using the VenorGeM Onestep kit (cat. no. 11-8050) according to the manufacturer's protocol (Minerva Biolabs GmbH) or by mycoplasma group-specific PCR (29).

Small-interfering RNA (siRNA) transfection. A total of $3x10^5$ cells (HBEC4KT, H1975, H2009 and H441) were transfected with predesigned siRNA (Silencer Select RNAi; cat. no. 4427037, Thermo Fisher Scientific, Inc.) to a final concentration of 6.25 nM using Lipofectamine® RNAiMAX (cat. no. 13778-150; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Knockdown of *GRHL2* was performed using two siRNA oligos, si-*GRHL2#*1 (cat. no. 4427037; ID, s36755; Thermo Fisher Scientific, Inc.) and si-*GRHL2#*2 (cat. no. 4427037, ID, s36756; Thermo Fisher Scientific, Inc.). The negative control used was Silencer™ Select Negative Control No. 1 siRNA (siNC; cat. no. 4390844; Thermo Fisher Scientific, Inc.). All transfections were performed at 37˚C in a humidified 5% CO_2 incubator. Durations of transfection were two days for cell proliferation and colony formation assays and four days for harvesting cells for RNA and protein extractions. Subsequent experimental procedures were performed immediately after completion of the transfections.

GRHL2 overexpression. A custom-ordered *GRHL2* expression vector, pLenti6/V5-GW/GRHL2, was constructed by replacing the coding sequence of *LacZ* in a pLenti6/V5-GW/lacZ vector (cat. no. K4955-10; Thermo Fisher Scientific, Inc.) with the *GRHL2* reference sequence (accession no. NM_024915.4), purchased from GenScript. $2x10^5$ of A549 cells plated in 6 cm dishes were transiently transfected with 1.5 ug of either the pLenti6/V5-GW/GRHL2 or the pLenti6/V5-GW/lacZ vector used as a control using Fugene®4K transfection reagent (cat. no. E5911; Promega Corporation) as aforementioned. Subsequently, 2 days of culture at 37˚C in a humidified 5% CO_2 incubator after the transfection, cells were harvested for protein or RNA extraction, or they were re-plated for proliferation or colony formation assays. Transfection efficiency was evaluated in $1x10^5$ A549 cells plated in 6-well plates by co-transfecting with 0.32 μg EGFP-expressing vector pEGFP-N3 (cat. no. 6080; Clontech.) at the ratio of 1:1 to the pLenti6/V5-GW/GRHL2 or pLenti6/V5-GW/lacZ vector (cat. no. K4955-10, Thermo Fisher Scientific, Inc.).

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Four days after transfection, total RNA was extracted from the cells (HBEC4KT, H1975, H2009 and H441) using the RNeasy mini kit (cat. no. 74106; Qiagen GmbH) following the manufacturer's protocol. Reverse transcription was completed using the SuperScript IV First-Strand Synthesis System with a random primer system (cat. no. 18091050; Thermo Fisher Scientific, Inc.) Using the PowerUp™ SYBR™ Green Master Mix (cat. no. A25742; Thermo Fisher Scientific, Inc.), qPCR was performed for *E-cadherin*, *VIMENTIN*, *TWIST1*, *Zinc finger E-box-binding homeobox 1 (ZEB1)*, *snail homolog* (*SNAI)1* and SNAI2. The thermocycling conditions

were follows: 95˚C for 20 sec, 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec. The melt condition was follows; 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec. *GAPDH* was used as an internal control, and the relative expression level was calculated by the ΔΔCq method (30). The primer sequences are shown in Table SI.

Cell proliferation assays. In total, 2 days after the transfection of siRNA, the cells (HBEC4KT, H1975, H2009, or H441) were plated into 96-well plates at 5,000 cells/well. Subsequently, 2 days later, a colorimetric proliferation assay was performed using the Cell Counting Kit-8 (CCK-8: cat. no. CK04; Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. Cells were incubated at 37˚C, 5% CO_2 for 2 h, measured absorbance at 450 nm and at 600 nm as a reference wavelength.

Colony formation assay. In total, 2 days after siRNA transfection, the cells (H441, H1975, H2009, and HBEC4KT) were replated into six-well plates at 1,000 cells/well for *GRHL2* silencing experiments. After 10 days of culture at 37°C, 5% CO_2, the colonies were stained with a solution containing 0.5% methylene blue tetrahydrate (cat. no. 137-06982; FUJIFILM Wako Pure Chemical Corporation) and 50% ethanol for fixation) at room temperature for 30 min. Colonies >1 mm in diameter were counted manually.

Western blot analysis. Western blotting was conducted as described previously using whole cell lysates (31). Lysate of transfected cells was collected 4 days after siRNA transfection and 2 days after *GRHL2* overexpression. Cell lysate of untransfected normal and lung cancer cell lines was collected at ~80% confluency. The primary antibodies used were rabbit anti-GRHL2 (cat. no. HPA062839; Merck KGaA; 1:500), mouse anti-E-cadherin (cat. no. 610181; BD Biosciences; 1:1,000), mouse anti-vimentin (cat. no. HPA001762; Merck KGaA; 1:500), rabbit anti-AKT (pan; cat. no. 4691; Cell Signaling Technology, Inc.; 1:1,000), rabbit anti-phosphorylated (p-)-AKT (ser473; cat. no. 4060; Cell Signaling Technology, Inc.; 1:1,000), rabbit anti-p44/42MAPK (Erk1/2; cat. no. 9102; Cell Signaling Technology, Inc.; 1:1,000), rabbit anti-p-p44/42MAPK (Erk1/2; cat. no. 4370; Cell Signaling Technology, Inc.; 1:1,000) and mouse anti-β-actin (cat. no. A2228; Merck KGaA; 1:2,000). The β-actin protein level was used as a control for protein loading. The primary antibodies were incubated at 4˚C overnight. The secondary antibodies conjugated with horseradish peroxidase (HRP) were anti-rabbit (cat. no. NA934-1ML; Cytiva) and anti-mouse antibodies (cat. no. NA931-1ML; Cytiva) at a 1:2,000 dilution and incubated at room temperature for 1 h. Primary and secondary antibodies of GRHL2 were diluted by Can Get Signal™ (cat. no. NKB-101; Toyobo Life Science). The intensities of the bands were quantified with Fiji (v.2.9.0/1.53t) (https://imagej.net/software/fiji/) (32) or ImageJ (v.1.53k) (https://imagej.nih.gov/ij/index.html) (33), where values of intensities normalized to β-actin, AKT or ERK were shown below the corresponding band images in the figures.

DNA copy number analysis. To perform a DNA copy number analysis, whole-exome sequencing (dbGaP Study Accession: phs001823.v1.p1; https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs001823.v1.p1) (34). A total of 68 lung adenocarcinoma, 20 lung squamous cell carcinoma and 63 non-small cell lung cancer (NSCLC)-histology not otherwise specified lung cancer cell lines were used (Table SII). Copy number variation (CNV) was determined using the R package 'DNAcopy' (R version 3.6, r-project.org/; DNAcopy version 1.36, https://bioconductor.org/packages/release/bioc/html/DNAcopy. html) with several modifications.

Choice of diploid controls. Because of differences in target capture enrichment during whole-exome sequencing, diploid controls were selected from the same batch as tumor samples. These batches were determined computationally by estimating the amount of noise (deviation from copy number segments) for each pair of tumor and control samples, followed by the hierarchical clustering of these noise values. Typically, tumor-control pairs from the same batch will have noticeably lower noise values. Next diploid controls were compared to other controls (from the same batch) to generate CNV segmentation plots. This allowed a further selection of controls with no or few CNV.

Recalibration. Because tumor samples are not diploid and frequently do not have an equal amount of copy number gains and losses, copy numbers were recalibrated by the visual inspection of each plot. In general, the lowest segments were set to one copy [$\log_2$(tumor/control)=-1], which shifted the subsequent segments so that $\log_2$(tumor/control) can be 0 (2 copies), 0.58 (3 copies), 1 (4 copies), and so on.

Statistical analysis. The Lung Cancer Explorer (LCE; http://lce.biohpc.swmed.edu/lungcancer/) was used to analyze the data from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/) and to perform meta-analyses on expression levels of *GRHL2* in tumor vs. normal tissues and impact of *GRHL2* expression on survival in patients with lung cancer. Univariate and multivariate analyses were performed using EZR (v. 1.55; jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html) (35). Pearson's correlation coefficients with associated P-values were calculated for correlation analyses by using EZR (v. 1.55). Statistically significant differences between two comparisons (control and one of the investigated groups) or >2 groups (control and multiple of the investigated groups) were analyzed by unpaired t-test or one-way ANOVA with Dunnett's post hoc test respectively, using the SPSS software (v.28; IBM Corp.). $P<0.05$ were considered to be statistically significant difference.

Results

GRHL2 expression is upregulated in lung cancer but is not associated with patient survival. To investigate the *GRHL2* expression profile in lung cancer and its impact on patient survival, statistical analyses were performed using LCE (23), with focus on a dataset of TCGA (cancer.gov/ccg/research/genome-sequencing/tcga). *GRHL2* expression was revealed to be significantly upregulated in both lung adenocarcinoma and squamous cell carcinoma tissues compared with that in normal lung tissues (Fig. 1A) in TCGA dataset. This result was confirmed by a meta-analysis of six datasets, including TCGA. *GRHL2* expression in adenocarcinoma

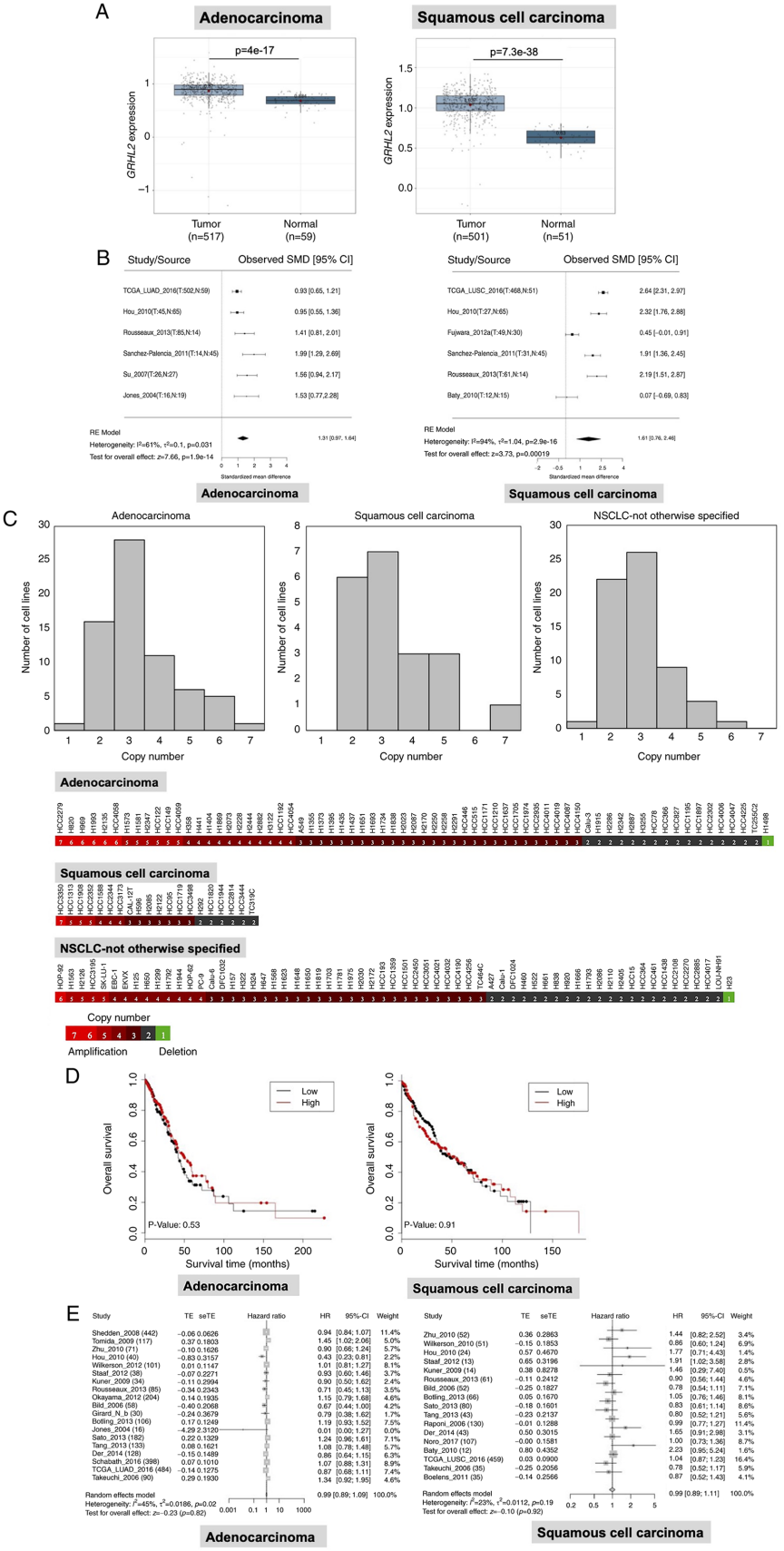

Figure 1. GRHL2 is expressed at high levels in lung cancer. (A) Expression analysis of GRHL2 in lung adenocarcinoma and squamous cell carcinoma in TCGA dataset. (B) Meta-analysis of GRHL2 expression in lung adenocarcinoma and squamous cell carcinoma from six studies. T, tumor, N, normal, RE, random effects. (C) DNA copy number analysis of GRHL2 showing frequent increases in the copy number of GRHL2 in NSCLC cell lines. (D) Kaplan-Meier survival curves for patients with high or low GRHL2 expression (the cut-off value is the median expression level). (E) Survival meta-analysis for GRHL2 in adenocarcinoma (left) and squamous cell carcinoma (right). In each forest plot, the name of each study is followed by the number of total tumor samples. GRHL2, Grainyhead-like 2; SMD, standardized mean difference; TE, estimated treatment effect; seTE, standard error of treatment effect; HR, hazard ratio; CI, confidence interval, TCGA, The Cancer Genome Atlas; NSCLC, non-small cell lung cancer.

Table I. Univariate and multivariate analyses of cox multivariate regression analysis in adenocarcinoma in TCGA cohort.

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
Sex						
Female	Reference			Reference		
Male	0.9058	0.632-1.298	0.5903	0.8083	0.5362-1.219	0.3097
Age, years						
>65	Reference			Reference		
≤65	0.7696	0.5353-1.107	0.1575	0.6787	0.4589-1.004	0.0522
Smoking status						
Never	Reference			Reference		
Former	0.9214	0.5095-1.666	0.7864	1.2300	0.6490-2.332	0.5255
Current	0.7176	0.3668-1.404	0.3324	0.7643	0.3741-1.561	0.4609
Stage						
I	Reference			Reference		
II	2.516	1.582-4.002	<0.0001[a]	3.0790	1.8620-5.091	<0.0001[a]
III	4.426	2.774-7.062	<0.0001[a]	4.8250	2.9390-7.922	<0.0001[a]
IV	3.266	1.665-6.407	0.0006[a]	4.4210	2.1770-8.981	<0.0001[a]
GRHL2 mRNA						
Low	Reference			Reference		
High	0.9162	0.6413-1.309	0.1575	0.9025	0.6140-1.327	0.6018

[a]P<0.05. GRHL2 expression levels were classified as high and low with median as the cut-off value. HR, hazard ratio; GRHL2, grainyhead-like 2; CI, confidence interval.

and squamous cell carcinoma tissues was 1.31 and 1.61-fold higher compared with that in normal tissue, respectively (Fig. 1B). In addition, copy number analysis by whole-exome sequencing, carried out as part of a different study (34), revealed increases in *GRHL2* copy numbers. Specifically, 51/68 (75.0%) adenocarcinoma, 14/20 (70.0%) squamous cell carcinoma and 40/63 (63.5%) NSCLC-not otherwise specified cell lines had > three *GRHL2* copies (mean ± SD; 3.35±1.25 in adenocarcinoma, 3.35±1.31 in squamous cell carcinoma and 2.94±0.97 in NSCLC-otherwise specified cell lines; Fig. 1C). These data suggest that the increased *GRHL2* expression may have resulted from gene amplification. Subsequently, it was investigated whether *GRHL2* expression was associated with patient survival in patients with adenocarcinoma or squamous cell carcinoma. Kaplan-Meier analysis in TCGA dataset revealed that overall survival was not significantly different in the *GRHL2* high- and low-expression adenocarcinoma and squamous cell carcinoma groups, following division using the median expression value (Fig. 1D). Univariate and multivariate Cox regression analysis in TCGA dataset showed that *GRHL2* expression was not associated with survival in patients with either adenocarcinoma (Table I) or squamous cell carcinoma (Table II). In addition, the absence of correlation between *GRHL2* expression between patient survival in lung cancer was also confirmed by a metanalysis using LCE (Fig. 1E). These data suggest that the expression of *GRHL2* was upregulated in both adenocarcinoma and squamous cell carcinoma, but this phenomenon was not associated with patient survival.

GRHL2 expression is associated with the epithelial phenotype in lung cancer. The association between GRHL2 protein expression and the EMT status in lung cancer was next investigated by evaluating the expression levels of GRHL2, E-cadherin (an epithelial marker) and vimentin (a mesenchymal marker) in a panel of lung cancer cell lines (HCC827, HCC4006, HCC4017, A549, H460, H441, H661, H1792, H1975, and H2009). As shown in Fig. 2A and B, expression levels of GRHL2 were not significantly positively correlated with E-cadherin (Pearson's correlation coefficient=0.059; P=0.067) or negatively correlated with Vimentin (Pearson's correlation coefficient=0.335; P=0.344). The correlation among the expression levels of *GRHL2*, *E-cadherin*, *vimentin* and master EMT transcription factors *ZEB1*, *ZEB2*, *SNAI1*, *SNAI2*, *TWIST1* and *TWIST2*, was then investigated using LCE. It was shown that *GRHL2* expression was positively and negatively correlated with *E-cadherin* and *vimentin*, respectively, in both patients with adenocarcinoma and patients with squamous cell carcinoma (Fig. 2C and D; Table SIII). This suggests that *GRHL2* expression is associated with the epithelial phenotype in lung cancer. In addition, *GRHL2* expression was found to be negatively correlated with five of the six (except *SNAI2*) of the master EMT transcription factors (Fig. 2C and D; Table SIII).

GRHL2 silencing induces a partial EMT in an hTERT/Cdk4-immortalized normal lung epithelial cell line without affecting growth. Next, to evaluate the impact of *GRHL2* silencing on EMT in normal lung epithelial

Table II. Univariate and multivariate analyses of cox multivariate regression analysis in squamous cell carcinoma in TCGA cohort.

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	P-value	HR	95% CI	P-value
Sex						
Female	Reference			Reference		
Male	1.038	0.7188-1.498	0.8434	1.0120	0.6924-1.478	0.9528
Age, years						
>65 years	Reference			Reference		
≤65 years	0.767	0.5447-1.080	0.1286	0.6727	0.4708-0.9611	0.0294
Smoking status						
Never	Reference			Reference		
Former	0.4628	0.1695-1.264	0.1327	0.4100	0.1462-1.150	0.0903
Current	0.5929	0.2114-1.663	0.3205	0.5453	0.1894-1.570	0.2611
Stage						
I	Reference			Reference		
II	1.202	0.8161-1.771	0.3514	1.1420	0.7654-1.704	0.5150
III	1.499	0.9979-2.251	0.0512	1.6030	1.060-2.424	0.0254
IV	2.042	0.6373-6.542	0.2295	1.8550	0.5745-5.992	0.3014
GRHL2 mRNA						
Low	Reference			Reference		
High	1.008	0.7317-1.387	0.963	0.9540	0.6845-1.330	0.7812

GRHL2 expression levels were classified as high and low with median as the cut-off value. HR, hazard ratio; CI, confidence interval; GRHL2, grainyhead-like 2.

cells, knockdown experiments were performed using the *hTERT*/*Cdk4* immortalized normal lung epithelial cell line HBEC4KT (28). This cell line does not harbor genetic alterations that may affect EMT phenotypes (28). *GRHL2* silencing was found to decrease E-cadherin whilst increasing vimentin expression, with upregulation in expression also observed in master EMT transcription factors *TWSIT1* (statistically significant) and *ZEB1* (not statistically significant; Fig. 3A and B). These suggest that partial EMT, characterized by decreased E-cadherin and increased Vimentin, albeit without completely switching their expression, has occurred (36). To evaluate the effects of this partial EMT on cell proliferation and colony formation, CCK-8 and colony formation assays were performed but no significant differences could be identified except for increased viability in si-*GRHL2*#2-treated cells (Fig. 3C and D). Therefore, these data suggest that *GRHL2* silencing promoted partial EMT in this cell line, but it did not significantly affect cell proliferation.

GRHL2 silencing induces partial EMT in lung cancer with cell type-dependent effects on growth. Next, three lung adenocarcinoma cancer cell lines H1975, H2009 and H441 were selected for evaluating the effects of *GRHL2* silencing on EMT status and growth. H1975 [*TP53* (*Arg273His*) and *EGFR* (*Thr790Met*, *Leu858Arg*) mutant) and H2009 (*TP53* (*Arg273Leu*) and *KRAS* (*Gly12Ala*) mutant] were selected due to the expression of significant levels of GRHL2 protein (Fig. 2A) and due to the possession of two major representative lung adenocarcinoma driver mutations (37,38). In addition, H441 [*TP53* (*Arg158Leu*) and *KRAS* (*Gly12Val*) mutant] was selected for experimentation because it expressed the highest levels of GRHL2 protein among the lung cancer cell line panel tested (Fig. 2A). *GRHL2* silencing did not alter E-cadherin protein or mRNA expression in any of the three aforementioned cell lines (Fig. 4A). By contrast, increased vimentin protein and mRNA expression levels in H1975 and H2009 cells were observed. However, *GRHL2* silencing significantly increased vimentin mRNA expression but did not affect protein expression in H441 cells (Fig. 4A and B). *GRHL2* silencing resulted in the upregulation of *ZEB1* expression in H1975, *TWIST1* expression in H2009 and *SNAI2* in H2009 and H441, suggesting a partial EMT and variable response to this silencing (Fig. 4A and B). *GRHL2* silencing also increased cell viability in H1975 cells but not in either H2009 or H441 cells (Fig. 4C). The colony formation assay revealed a significant decrease in colony formation by H2009 cells, but no change was observed in H1975 cells (Fig. 4D). H441 cells did not form colonies and therefore could not be tested (Fig. 4D). These results suggest that *GRHL2* silencing induced EMT in the lung cancer cell lines, but its effects on proliferation and colony formation were cell type-dependent and varied greatly among the lung adenocarcinoma cell lines.

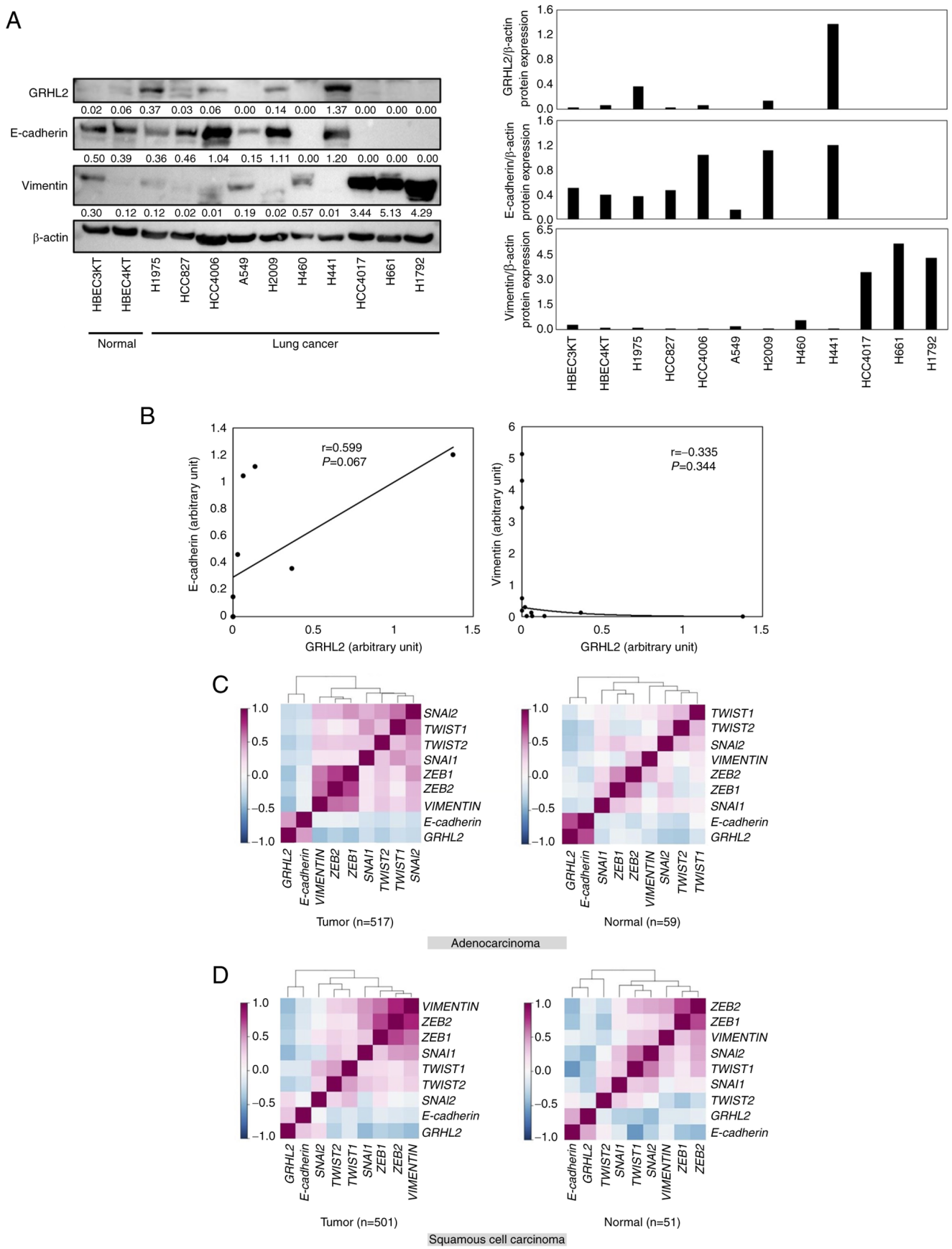

Figure 2. GRHL2 is associated with an epithelial phenotype in lung cancer. (A) Western blotting of GRHL2, E-cadherin and Vimentin in a panel of lung cancer and immortalized bronchial epithelial cell lines. Values below bands indicate the semi-quantitation of band intensities normalized to β-actin. (B) Correlations between bands intensities of GRHL2 and E-cadherin (left graph) or Vimentin (right graph) in (A). Correlation analysis between the expression of *GRHL2*, *E-cadherin*, *VIMENTIN* and six master epithelial-to-mesenchymal transition regulator genes in (C) adenocarcinomas and (D) squamous cell carcinomas in The Cancer Genome Atlas dataset. The degrees of positive or negative correlation, revealed by Pearson's correlation coefficients, are colored by the darkness of purple or blue, respectively. GRHL2, Grainyhead-like 2; ZEB, Zinc finger E-box-binding homeobox; SNAI, snail homolog.

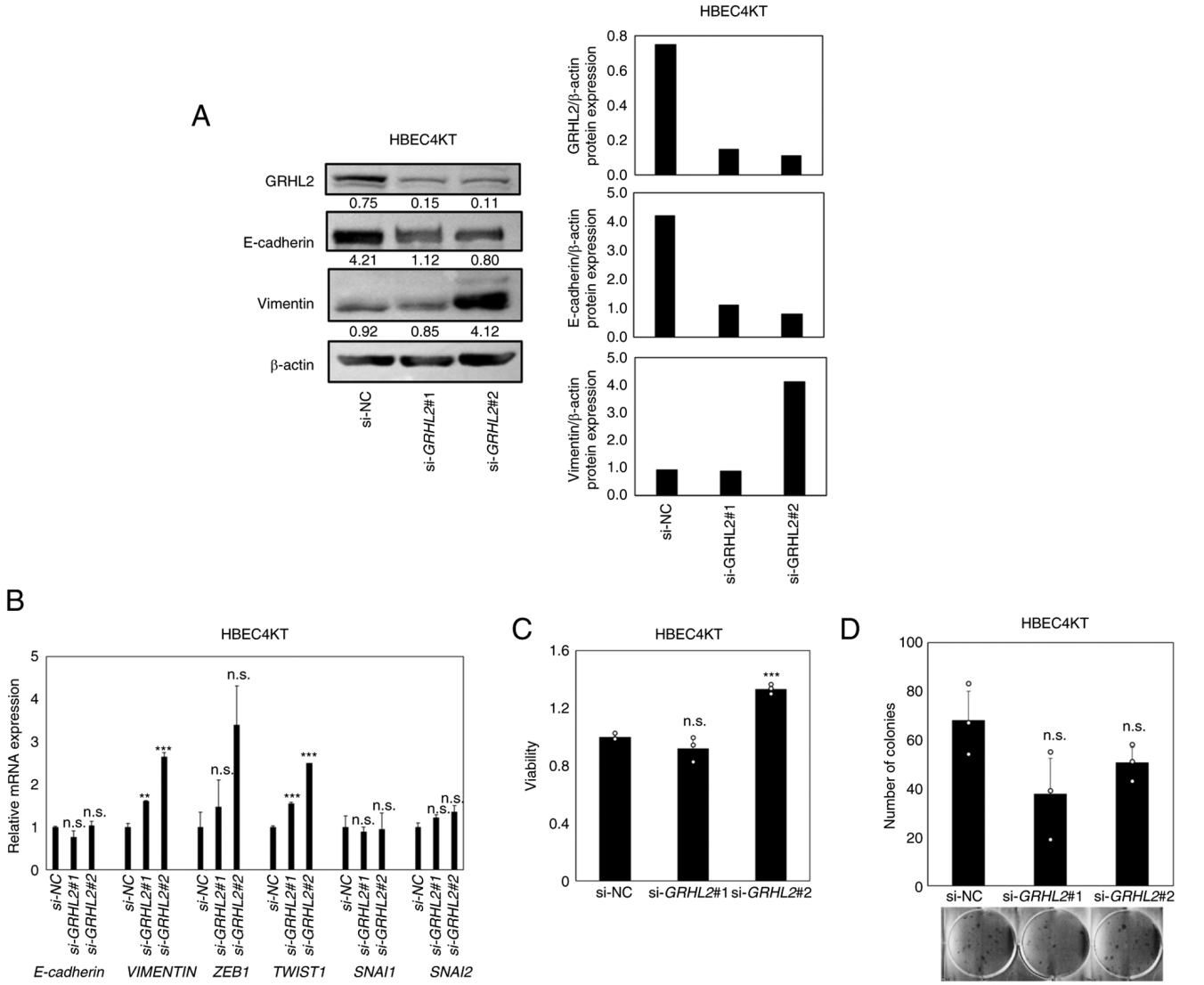

Figure 3. GRHL2 silencing induces a partial EMT in hTERT/Cdk4-immortalized normal lung epithelial cell line (HBEC4KT) without affecting its growth. (A) Western blotting of GRHL2, E-cadherin and Vimentin in the immortalized normal bronchial epithelial cell line HBEC4KT transfected with either control or GRHL2 siRNA. Values below bands indicate quantitation of band intensities normalized to β-actin. Quantification of band intensities is shown in the right graph. (B) Reverse transcription-quantitative PCR of E-cadherin, VIMENTIN and four master EMT regulators in HBEC4KT cells, transfected with either control (si-NC) or GRHL2 siRNA. (C) Proliferation and (D) colony formation assays for HBEC4KT cells transfected with either control or GRHL2 siRNA. Results are shown as the mean ± SD (n=3), where comparisons were performed with one-way ANOVA followed by the Dunnett test. **P<0.01 and ***P<0.001 vs. si-NC. GRHL2, Grainyhead-like 2, siRNA, small-interfering RNA; NC, negative control; n.s., non-significant; EMT, epithelial-to-mesenchymal transition; ZEB, Zinc finger E-box-binding homeobox; SNAI, snail homolog.

GRHL2 silencing promotes the phosphorylation of AKT and ERK. Compared with the present study, numerous studies have reported that EMT can promote cancer cell proliferation (39-41). Additionally, several studies have found oncogenic roles in *GRHL2* by showing growth suppression in several cancer cell types, including breast, pancreatic and colorectal cancers, after *GRHL2* silencing (5,6,11,16,18,20). Growth suppression induced by *GRHL2* silencing occurred through inhibition of the AKT or ERK pathways (18,20). This finding that *GRHL2* silencing causes the inhibition of AKT and ERK is inconsistent with the function of *GRHL2* as an EMT suppressor because the AKT and ERK pathways are associated with EMT in various types of canceer including lung and colorectal cancers (42-44). It was therefore hypothesized that the differential effects of *GRHL2* silencing on growth may be due to whether the AKT or ERK pathway was induced or suppressed by *GRHL2* silencing. Therefore, the effect of *GRHL2* silencing on the phosphorylation of AKT and ERK was next assessed in HBEC4KT, H1975, H2009 and H441 cell lines by western blotting. *GRHL2* silencing enhanced the phosphorylation of both AKT and ERK in HBEC4KT, H1975 and H2009 cell lines, but it did not affect either AKT or ERK in H441 (Fig. 5). The finding that both p-AKT and p-ERK were equally upregulated in HBEC4KT, H1975 and H2009 cells was unexpected, because the effects of *GRHL2* silencing in these cells further downstream differed substantially. This suggests that the differential effects of *GRHL2* silencing on cell proliferation among normal bronchial epithelial and lung cancer cells do not occur through the differences in the phosphorylation of the AKT or ERK pathways.

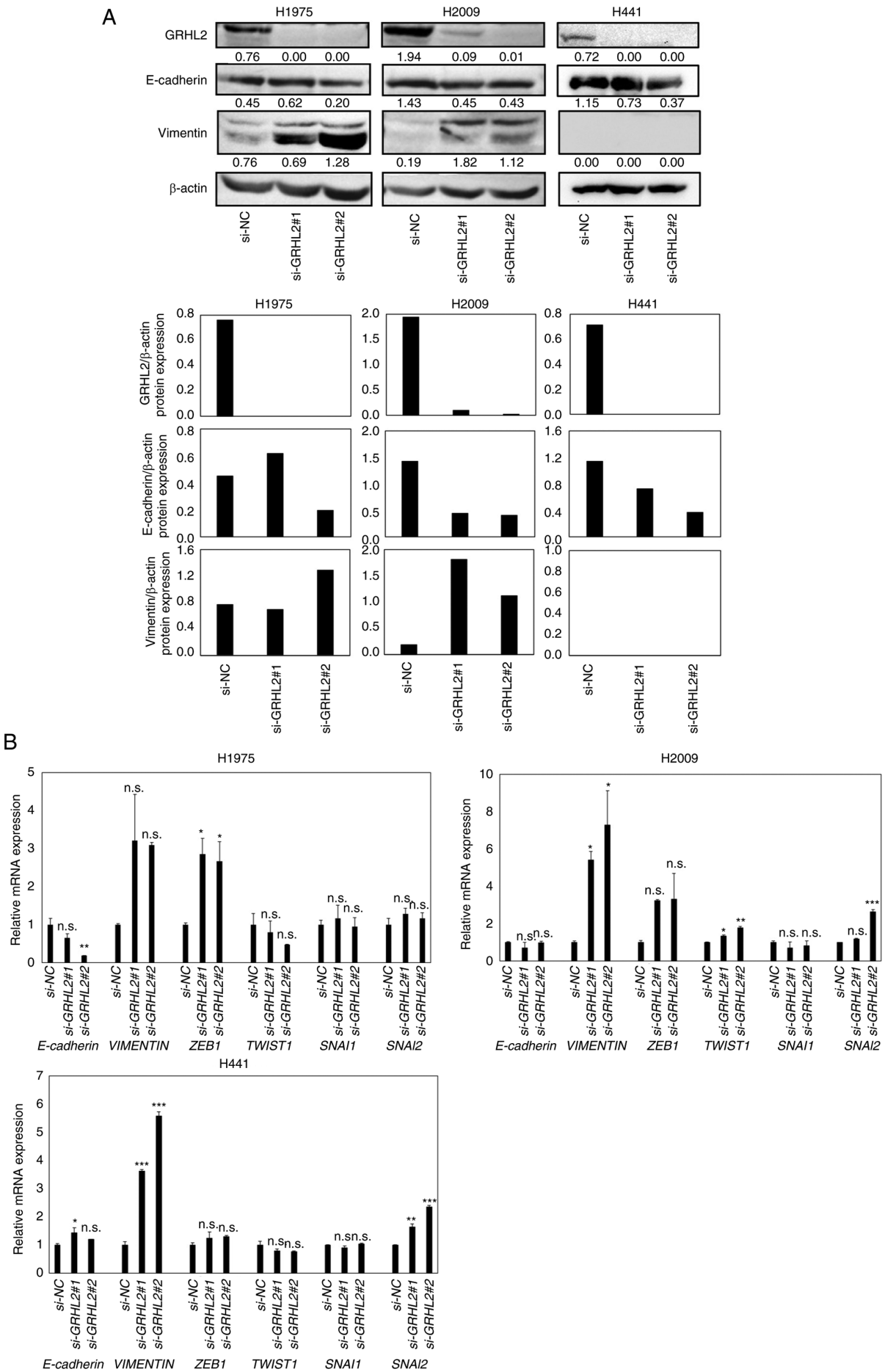

Figure 4. Continued.

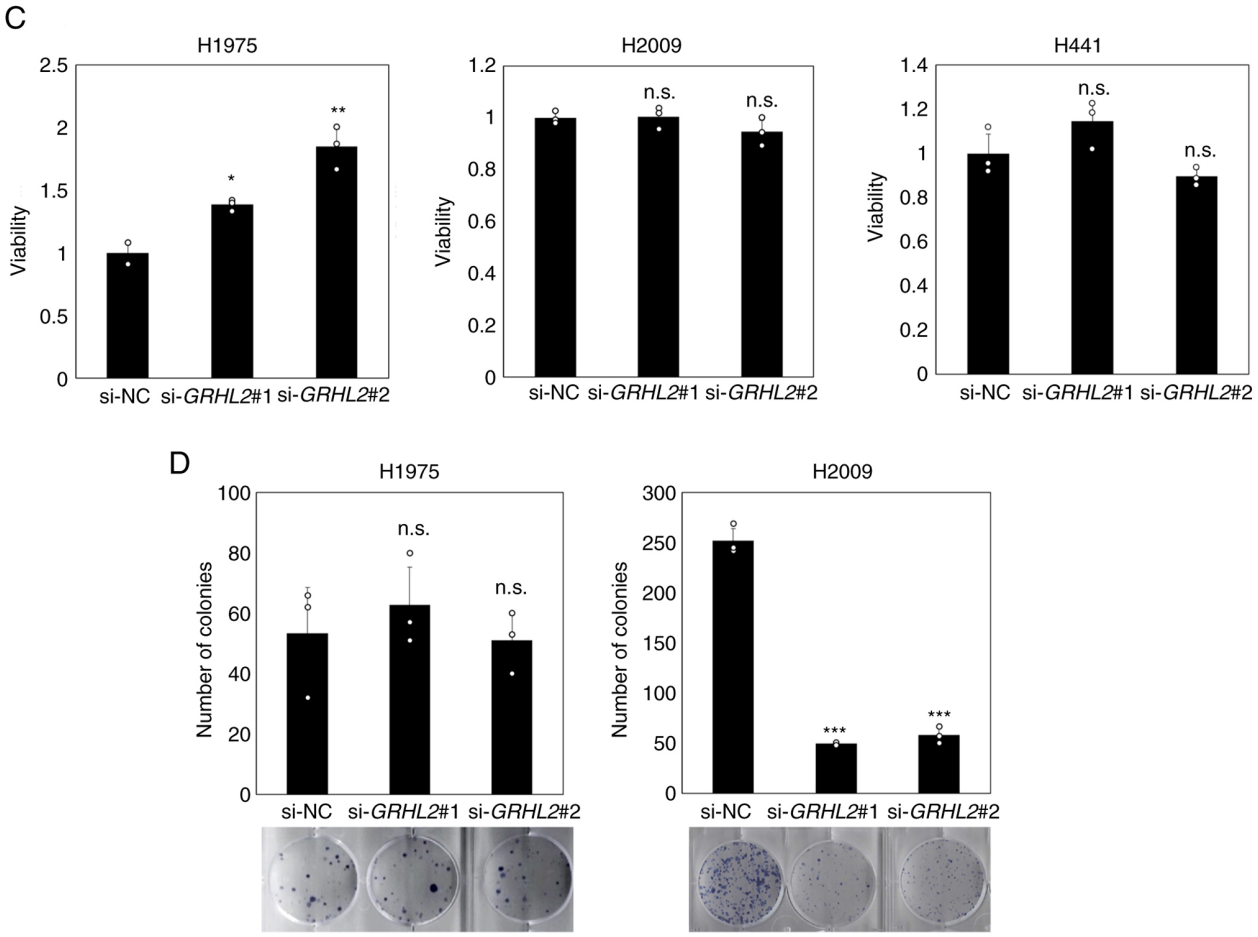

Figure 4. GRHL2 silencing induces a partial EMT in lung cancer but its effects on growth are cell type-dependent. (A) Western blotting of GRHL2, E-cadherin and vimentin in the lung cancer cell lines H1975 and H2009 transfected with control or GRHL2 siRNA. Values below bands indicate quantitation of band intensities normalized to β-actin. Quantification of band intensities is shown in the bottom graph. (B) Reverse transcription-quantitative PCR of E-cadherin, vimentin and four master EMT regulator genes in H1975 and H2009 cells transfected with control (si-NC) or GRHL2 siRNA. (C) Proliferation and (D) colony formation assays of H1975 and H2009 cells transfected with either control or GRHL2 siRNA. Results are shown as the mean ± SD, assessed by one-way ANOVA followed by the Dunnett test. *P<0.05, **P<0.01 and ***P<0.001 vs. si-NC. GRHL2, Grainyhead-like 2; siRNA, small-interfering RNA; NC, negative control; n.s., non-significant; EMT, epithelial-to-mesenchymal transition; ZEB, Zinc finger E-box-binding homeobox; SNAI, snail homolog.

GRHL2 overexpression does not affect the proliferation in A549 cells. To evaluate the effects of *GRHL2* overexpression in lung cancer cells, *GRHL2* overexpression experiments were performed using A549 cells [*TP53* wild-type, *KRAS*(*Gly12Ser*) and *liver kinase B1* (*Gln37Ter*) mutant], which lacked detectable expression levels of the GRHL2 protein (Fig. 2A). In total, ~50% transfection efficiency was confirmed by co-transfection with the *EGFP*-expressing vector pEGFP-N3 (Fig. S1). Overexpression of the GRHL2 protein was achieved in the A549 cells transfected with the *GRHL2*-expressing vector (Fig. 6A). *GRHL2* overexpression reduced the protein expression of E-cadherin, whilst the protein expression of vimentin remained unaltered (Fig. 6A). *GRHL2* overexpression decreased the mRNA expression of *E-cadherin* and the increased mRNA expression of *ZEB1* and *SNAI1* without affecting *TWIST1* or *SNAI2* expression (Fig. 6B). However, *GRHL2* overexpression did not affect A549 proliferation (Fig. 6C).

Discussion

Both oncogenic and tumor suppressive roles of *GRHL2* have been reported in various malignancies (7-21). A study previously showed that *GRHL2* is overexpressed in lung cancer, which is in turn associated with poor prognosis. In addition, silencing *GRHL2* expression was found to suppress proliferation and colony formation, but it also promoted migration and invasion in lung cancer cell lines (12). Based on the finding that *GRHL2* silencing promoted migration and invasion in a lung cancer cell line H1299, this previous study concluded that *GRHL2* primarily functions as a tumor suppressor by suppressing metastasis (12). However, this previous study also presented data suggesting tumor-promoting roles of *GRHL2*, including that higher expression levels of GRHL2 in lung cancer samples are associated with poorer patient prognosis. In addition, suppressed proliferation and colony formation resulted from GRHL2 silencing (12). Therefore, the roles of GRHL2 in lung cancer remain unclear. In the present study, increased expression of *GRHL2* was observed in lung cancer tissues without association with patient survival. Expression data from a public database together with the experimental results of the present study indicated that *GRHL2* likely serves an important role in maintaining an epithelial phenotype in lung cancer. Nevertheless, despite its EMT-suppressing function in lung cancer, it was shown that *GRHL2* silencing

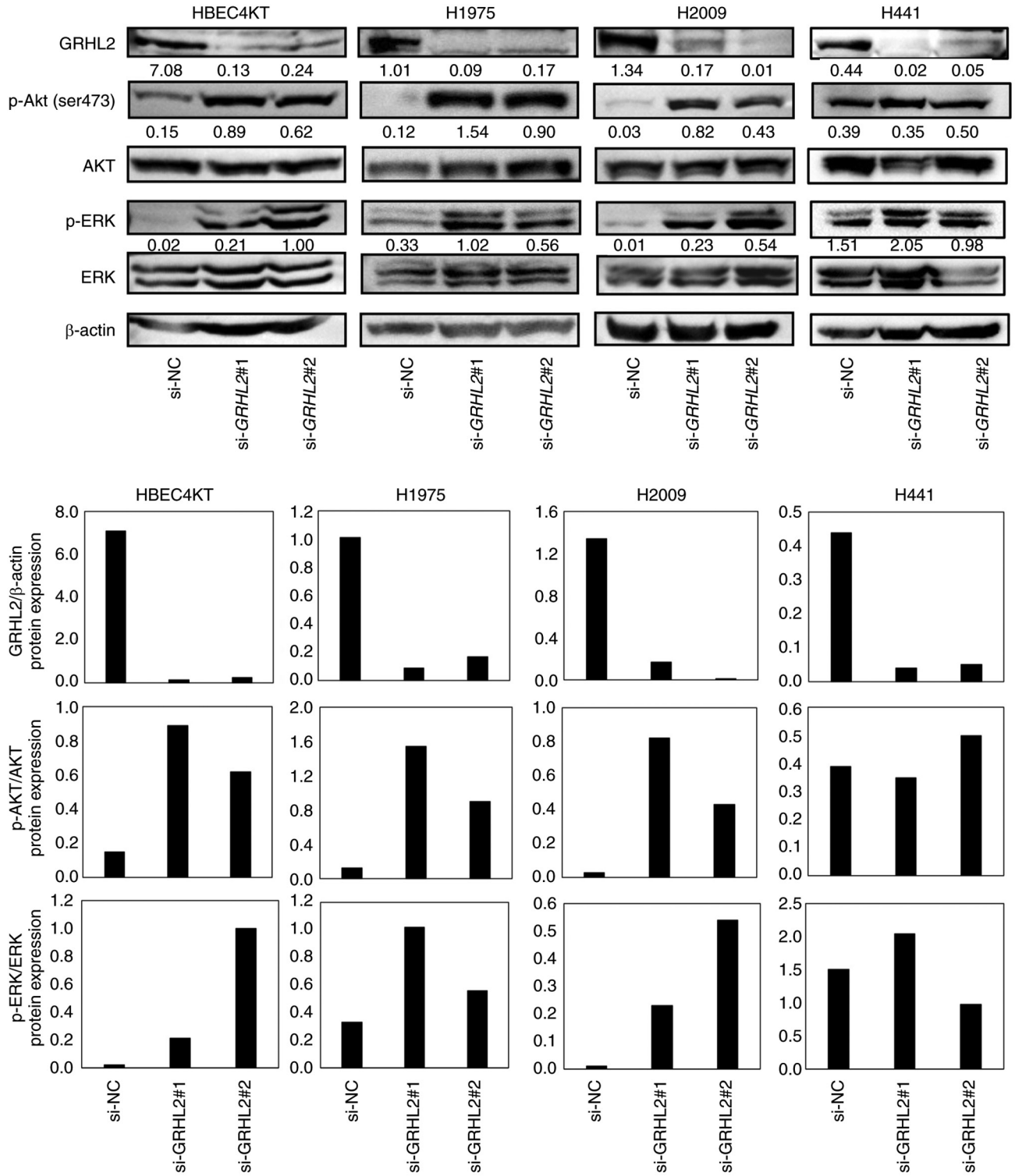

Figure 5. GRHL2 knockdown promotes the phosphorylation of AKT and ERK in HBEC4KT and lung cancer cell lines. Western blotting of GRHL2, p- or total AKT and p- or total ERK in HBEC4KT, H1975 and H2009 cells transfected with control or GRHL2 siRNA. Values below bands indicate the semi-quantitation of band intensities normalized to AKT for p-AKT (ser473), ERK for p-ERK or β-actin for GRHL2. GRHL2, Grainyhead-like 2; siRNA, small-interfering RNA; NC, negative control; p-, phosphorylated.

resulted in either growth-promoting or -suppressing effects in lung cancer cell lines.

Consistent with a previous study (12), it was shown that *GRHL2* is overexpressed in lung cancer in a meta-analysis of gene expression datasets. In addition, this previous study also showed that patients with lung cancer and higher protein expression of GRHL2 had significantly shorter survival (12). However, the meta-analysis in the present study revealed the absence of association between *GRHL2* expression and patient survival in lung cancer, suggesting that *GRHL2* expression is not associated with survival in patients with lung cancer. This was unexpected because of the apparent EMT-suppressing

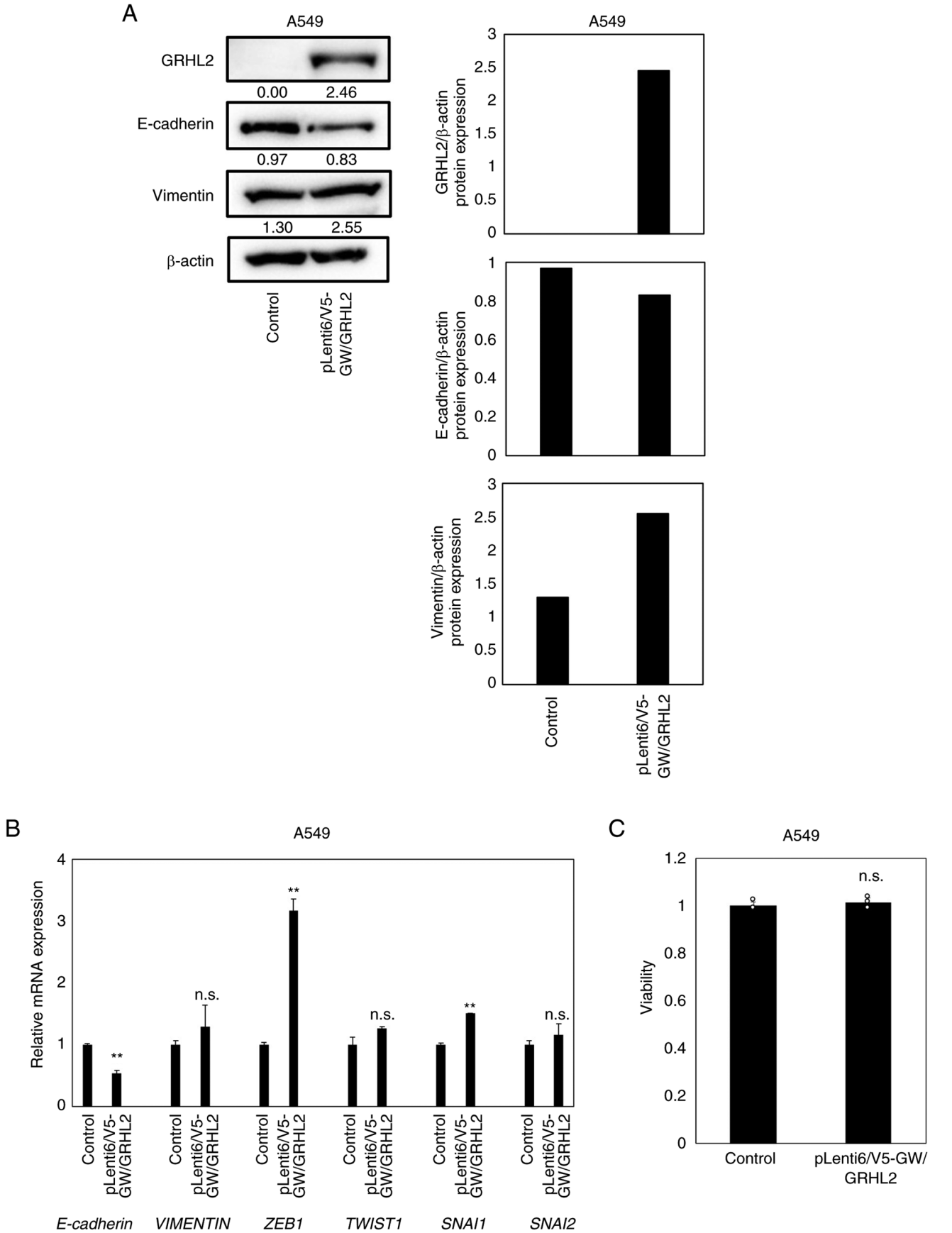

Figure 6. GRHL2 overexpression does not affect proliferation in A549 cells. (A) Western blotting of GRHL2, E-cadherin and Vimentin in the lung cancer cell line A549 transiently transfected with GRHL2- or LacZ-expressing vectors. Quantification of band intensities is shown in the right graph. (B) Reverse transcription-quantitative PCR of *E-cadherin*, *vimentin* and four master epithelial-to-mesenchymal regulator genes in A549 transiently transfected with *GRHL2*- or *LacZ*-expressing (control) vectors. **P<0.01 vs. control. (C) Proliferation assay for A549 transiently transfected with *GRHL2*- or *LacZ*-expressing vectors. Results are shown as the means ± SD (n=3), where comparisons with controls were performed by the unpaired t-test. n.s., indicates 'no significant difference'. GRHL2, Grainyhead-like 2; ZEB, Zinc finger E-box-binding homeobox; n.s., not significant; SNAI, snail homolog.

function of GRHL2. Bi-directional effects of *GRHL2* on the growth of lung cancer cells were observed, which likely explains the reason underlying this ambiguous result.

GRHL2 is one of the several transcription factors that can suppress EMT (3,6). However, the predominant roles that *GRHL2* plays in maintaining the epithelial phenotypes in lung cancer remain to be clarified. The present study suggested an association between *GRHL2* expression and epithelial phenotypes was identified in a panel of lung cancer cell lines and clinical tumors tissues. Additionally, *GRHL2* silencing induced partial EMT in lung cancer cell lines and an immortalized normal bronchial epithelial cell line. These results suggest an important role of *GRHL2* in maintaining an epithelial phenotype in lung cancer.

Despite the partial EMT induced by *GRHL2* silencing in the lung cancer cell lines, its impact on growth differed substantially among the cell lines tested, as shown by enhanced viability in H1975 and inhibited clonogenic growth in H2009. To gain insights into the interpretation of these results, the phosphorylation of AKT and ERK was investigated because these growth-promoting proteins were previously found to be suppressed by *GRHL2* silencing (18,20). *GRHL2* silencing resulted in increased levels of p-AKT and p-ERK in all cell lines studied, suggesting that the differential activation of either AKT or ERK may not be attributable to the observed differences in the effects of *GRHL2* silencing on cell proliferation. Notably, one previous study (20) reported that *GRHL2* silencing suppressed the proliferation of two colorectal cancer cell lines through decreased AKT phosphorylation. However, in the present study, it was shown that *GRHL2* silencing resulted in the increased activation of AKT even in the H2009 lung cancer cell line, which in turn inhibited colony formation (Fig. 4D). These findings suggested that the effects of *GRHL2* on the AKT or ERK pathways markedly vary among cancer types. Therefore, the effects of *GRHL2* silencing on the phosphorylation status of AKT and ERK should be evaluated in a variety of human cancer cell lines, such as breast and pancreatic cancer. ERK can serve a pro-apoptotic function when it is translocated into the mitochondria instead of the nucleus (45). Therefore, it remains possible that upregulated ERK activity may not promote, but rather suppress, cell proliferation in the experiments performed in the present study.

A discrepancy was observed in the results of cell viability and colony formation assays in H2009 cells, whereby *GRHL2* silencing did not affect proliferation but inhibited colony formation. It was hypothesized that these contradictory results could be explained by the differences in the properties of cancer cells these two assays evaluate. The viability assay evaluates cell proliferation, whereas colony formation assays measure the ability of individual cells to survive and propagate over long periods of time, typically over 10-14 days (46,47). Notably, a previous study (46) described that colony formation assays can be used to evaluate self-renewing capacity *in vitro*. Relevant roles of *GRHL2* in self-renewal have been reported (48,49). The growth suppression induced by *GRHL2* silencing in the present study was only observed in colony formation but not in viability assays in H2009, suggesting the involvement of *GRHL2* in cell stemness. Collectively, results of the present study in H2009 cells suggest that *GRHL2* may confer anti-apoptotic and/or self-renewing abilities. Therefore, this hypothesis warrants further investigation in future studies.

GRHL2 overexpression did not affect proliferation in A549 cells, which lacked detectable expression levels of the endogenous GRHL2 protein. Both growth-promoting and -suppressive effects of *GRHL2* overexpression have been reported in numerous types of different cell models (8,10,13-16,20). To further evaluate the effects of *GRHL2* overexpression on proliferation and clonogenic growth in lung cells, *GRHL2* overexpression experiments using additional lung cancer cell lines and immortalized normal lung epithelial cells are planned as future experiments. In addition, it was an unexpected observation that *GRHL2* overexpression decreased the expression of E-cadherin on both mRNA and protein levels because of its function of positive transcription factor for E-cadherin (3). One possible explanation for this result is the increased *ZEB1* expression in A549 cell, which was also induced in response to *GRHL2* overexpression. It was possible that ZEB1 counteracted the function of *GRHL2* as a positive regulator of E-cadherin expression, leading to decreased levels of E-cadherin expression. To test this hypothesis, luciferase reporter assay is required for evaluating transcriptional activity of E-cadherin in *GRHL2*-overexpressing cells with or without *ZEB1* silencing.

Altogether, the present study revealed that *GRHL2* expression was associated with an epithelial phenotype, but its expression was not associated with prognosis in lung cancer. Gene silencing experiments suggested that *GRHL2* is not likely to be a definitive tumor suppressor or an oncogene, instead acting as either of them depending on the cell context.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NK and MS conceived and designed the study. NK, KM, KK, NM, MT, YT, YI, MY, NS and MS acquired data. NK, KM, LG, LC, YX, IT, MM, LG, JDM and MS analyzed and interpreted the data. LG and JDM provided technical or material support. NK, NS, LG, JDM and MS wrote, reviewed and revised the manuscript. All authors have read and approved

the final version of the manuscript. NK and MS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

JM receives royalties from the NIH and University of Texas Southwestern for distribution of human cell lines. The remaining authors declare that they have no competing interests.

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