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DEPDC1B enhances migration and invasion of non-small cell lung cancer cells via activating Wnt/β-catenin signaling

Yi Yang [a,b,1], Lei Liu [a,c,1], Junchao Cai [a,c], Jueheng Wu [a,c], Hongyu Guan [d], Xun Zhu [a,c], Jie Yuan [a,e], Mengfeng Li [a,c,*]

[a] Key Laboratory of Tropical Disease Control (Sun Yat-Sen University), Ministry of Education, Guangzhou, Guangdong, China
[b] Department of Pharmacology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, Guangdong, China
[c] Department of Microbiology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, Guangdong, China
[d] Department of Endocrinology and Diabetes Center, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong, China
[e] Department of Biochemistry, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, Guangdong, China

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ABSTRACT

Non-small cell lung cancer (NSCLC) remains a highly challenging and deadly malignancy with limited improvements in prognosis over years. Further understanding the molecular events involved in NSCLC oncogenesis and progression will help develop new and effective therapeutic strategies. In this study, we identified a ubiquitous up-regulation of DEPDC1B in NSCLC cell lines and clinical specimens, as well as its inverse correlation with patient survival. Ectopic expression of DEPDC1B endowed NSCLC cells with enhanced migration and invasion, while silencing its expression suppressed these traits. Mechanistic study showed that DEPDC1B was able to activate Wnt/β-catenin signaling, and that depletion of TCF4 or LEF1 abrogated the biological effects of DEPDC1B on cellular migration and invasion. Taken together, our data demonstrate that DEPDC1B might confer metastasis-related malignant phenotype to NSCLC in a Wnt/β-catenin dependent manner, providing new insights in developing novel anti-NSCLC strategies.

1. Introduction

Lung cancer is the most common type of cancer and the leading cause of cancer-related morbidity and mortality [1,2]. The most prevalent class within lung cancer, non-small cell lung cancer (NSCLC), accounting for approximately 85% of all lung cancer cases [3], has the overall five-year survival rate as low as 15% with all stages and subtypes combined [3]. During the past few decades, while advancements of NSCLC treatments, such as surgical therapy, radiotherapy, chemotherapy and recently developed molecular targeting therapy, have been made, the survival rates for the disease have not been significantly improved. For patients bearing stage IV NSCLC, for example, the 5-year survival rate is only approximately 1%, with median survival time of 7 months [4]. Identifying key molecules contributing to the malignant properties of NSCLC cells is essential for future development of new and effective anti-NSCLC approaches.

Distant metastasis of cancer contributes to more than 90% of cancer-related mortality [5]. In NSCLC, micrometastases can be found even at very early stage and have been associated with shorter disease-free survival, making the disease possibly a systemic disease regardless of the TNM staging system [6,7]. Biologically, tumor metastases can be initiated with a prominent event known as epithelial–mesenchymal transition (EMT), which is characterized by specific morphogenetic changes, loss of cell–cell adhesion and augmentation of cell motility [8,9]. It has also been demonstrated that the canonical Wnt/β-catenin signaling pathway plays an essential role in EMT as well as cancer metastasis [10,11], in addition to its involvement in cell fate determination during development and organogenesis [12]. Secreted Wnt ligands bind Frizzled and LRP5/6 transmembrane receptors to prevent degradation of β-catenin in the cytoplasm by sequestering it away from the destruction complex consisting of APC (the adenomatous polyposis coli gene product), glycogen synthase kinase-3β (GSK-3β) and Axin [13]. And consequently, β-catenin accumulates in the nucleus, bind

Abbreviations: NSCLC, non-small cell lung cancer; NLE, normal lung epithelial; KSFM, keratinocyte-SFM medium; UICC, Union for International Cancer Control; TNM, Tumor-Node-Metastasis; EMT, epithelial–mesenchymal transition; TCGA, The Cancer Genome Atlas; SCC, squamous cell carcinoma; ADC, adenocarcinoma; APC, the adenomatous polyposis coli gene product; GSK-3β, glycogen synthase kinase-3β; TCF, T cell factor; LEF, lymphoid enhancer-binding factor; NC, negative control.

* Corresponding author at: Sun Yat-Sen University, Zhongshan School of Medicine, 74 Zhongshan Road II, Guangzhou, Guangdong 510080, China. Fax: +86 20 87331209.
E-mail address: limf@mail.sysu.edu.cn (M. Li).
[1] Authorship note: Yi Yang and Lei Liu contributed equally to this work.

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TCF/LEF (T cell factor/lymphoid enhancer-binding factor) proteins, and regulate gene expression [12]. In NSCLC, nuclear localization of β-catenin has been demonstrated in patients [14], and hyperactive Wnt/β-catenin signaling has been reported to be associated with enhanced ability of tumor cells to infiltrate and colonize in the brain and bones [15]. However, mutations of β-catenin or APC, which are the major cause of aberrant activation of Wnt signaling in colorectal cancer, are rare in NSCLC [16], making it interesting to identify molecular mechanisms that mediate the activation of this pathway.

DEPDC1B is a recently identified gene localized at human chromosome 5q12.1, and has been reported to be associated with delayed cell death and increased cell proliferation in breast cancer. In this present study, we show that DEPDC1B is up-regulated in NSCLC in a reverse correlation with patient survival. We also find that DEPDC1B promotes tumor cell migration and invasion through activating Wnt/β-catenin signaling. Our data suggest that DEPDC1B might be a potentially valuable predictive factor for the prognosis, and a potential therapeutic target as well, for NSCLC.

2. Material and methods

2.1. Cell cultures

Primary normal lung epithelial cells (NLE) were cultured in the keratinocyte-SFM medium (KSFM) supplemented with 40 μg/ml bovine pituitary extract, 1.0 ng/ml EGF, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml gentamicin and 100 units/ml nystatin (Invitrogen, Carlsbad, CA) as previously described [17]. NSCLC cell lines, including NCI-H292, NCI-H358, NCI-H460, NCI-H596, NCI-H1299, A549, Calu-3 and SK-MES-1, were obtained from the Shanghai Institutes of Biological Sciences Cell Bank (Shanghai, China) or Fu Erbo Biotechnology Co., Ltd. (Guangzhou, China), and maintained in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen).

2.2. Tumor specimens from patients

Clinical tissue specimens used in this study were histopathologically and clinically diagnosed at the Sun Yat-Sen University Cancer Center from 2000 to 2004. For the use of these clinical materials for research purposes, prior patients' consents and approval from the Institutional Research Ethics Committee were obtained.

2.3. RNA extraction and real-time PCR

RNA extraction, RT, and real-time PCR were performed as we and others described previously [18–20]. Primers are listed in Supplemental Materials and Methods.

2.4. Plasmids and generation of stably engineered cell lines

DEPDC1B expression plasmid was generated by PCR subcloning human DEPDC1B coding sequence into retroviral transfer plasmid pSin-puro (Clontech, Palo Alto, CA) to generate plasmid pSin-DEPDC1B. For depletion of DEPDC1B, two human shRNA sequences were cloned into pSuper-retro-puro to generate pSuper-retro-DEPDC1B-sh1 and pSuper-retro-DEPDC1B-sh2, respectively, with sequences as the following: sh1: GGTACAAGCGTCACAGTAT; or sh2: CTGCTAGATTGGTAACGTT. Retroviral production and infection were performed as previously described [21]. The reporter plasmids containing wild-type (CCTTTGATC; TOPflash) or mutated (CCTTTGGCC; FOPflash) TCF/LEF DNA binding sites were purchased from the Upstate Biotechnology (Lake Placid, NY).

2.5. Wound healing, cell invasion and migration assays

These assays were performed as described in a previous report [22] and in Supplemental Materials and Methods.

2.6. Western blotting analysis

Western blotting analysis was performed as described previously [23], using antibodies against DEPDC1B (Sigma, St. Louis, MO), E-cadherin, γ-catenin, CK18, Vimentin, and N-cadherin (BD Biosciences, San Jose, CA), and anti-α-tubulin (Sigma, Saint Louis, MO, USA), respectively. All Western blotting assays were independently repeated for at least three times.

2.7. Luciferase reporter assay

Luciferase assay was carried out according to a standard method [24,25] (also see Supplemental Materials and Methods).

2.8. Immunohistochemistry assays (IHC)

By using primary antibody of DEPDC1B, IHC in 76 NSCLC tissue specimens were done and analyzed according to previous reports [20] (also see Supplemental Materials and Methods).

2.9. Statistical analysis

All statistical analyses were carried out using the SPSS 13.0 statistical software package. A cohort of 76 NSCLC patients were divided into two groups based on DEPDC1B expression level, namely, the low-DEPDC1B expression group (below the median value) and the high- DEPDC1B expression group (above the median value), for clinical survival analysis. The Kaplan–Meier method was used to establish survival curves, and log-rank test was applied for comparative analysis of differences in patient survival. The significance of various variables for survival was analyzed using the Cox proportional hazards model in the multivariate analysis. Comparisons between groups for statistical significance were performed with a two-tailed paired Student's t test. In all cases, $P < 0.05$ was considered statistically significant.

3. Results

3.1. DEPDC1B is up-regulated in NSCLC cell lines and tissues

Expression of DEPDC1B was determined in 8 human NSCLC cell lines, including NCI-H292, NCI-H358, NCI-H460, NCI-H596, NCI-H1299, A549, Calu-3, and SK-MES-1, and in primary normal lung epithelial (NLE) cells, using quantitatively real-time PCR and Western blotting analyses. As shown in Fig. 1A, all tested NSCLC cell lines displayed markedly increased expression of DEPDC1B, with a majority (seven) of the cell lines exhibiting more than 5-fold increase as compared with that in NLE. Moreover, DEPDC1B was also found up-regulated in 5 cancer specimens when compared with their paired adjacent non-cancerous lung tissue (Fig. 1B). Similar results were obtained by examining data from a larger cohort of NSCLC patients (90 paired) provided by The Cancer Genome Atlas (TCGA) project (http://cancergenome.nih.gov/) in Fig. 1C. Furthermore, expression of DEPDC1B protein was also determined by IHC in clinical specimens collected from 33 NSCLC patients, and the result showed elevated DEPDC1B levels in all stages of NSCLC as compared with normal lung tissue (Fig. 1D), suggesting that upregulation of DEPDC1B is a clinically relevant feature of NSCLC.

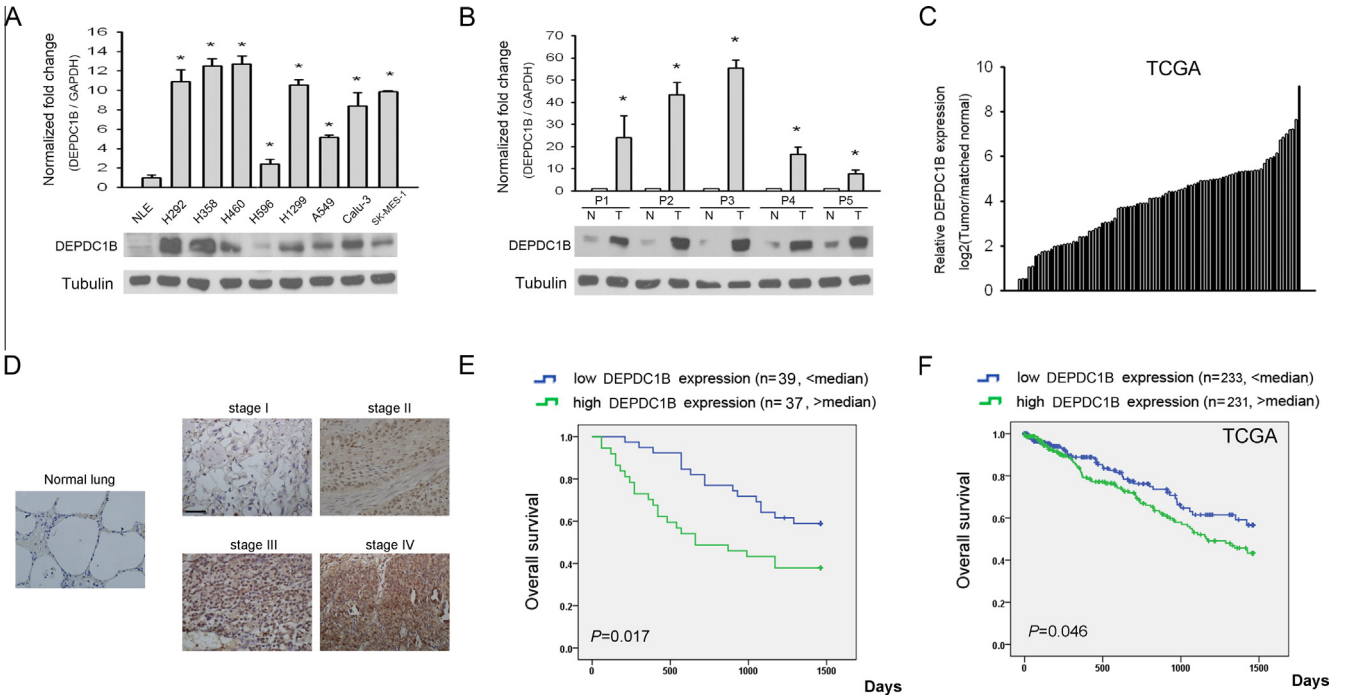

Fig. 1. DEPDC1B is up-regulated in NSCLC and indicates worse prognosis. (A) Expression of DEPDC1B mRNA (upper panel) and protein (lower panel) in benign primary lung epithelial cells (NLE) and indicated NSCLC cell lines. (B) Expression of DEPDC1B mRNA (upper panel) and protein (lower panel) in 5 NSCLC tumor specimens (T) and their paired adjacent non-cancerous lung tissue (N). For (A) and (B), error bars represent standard error of the mean (SEM) of 3 independent experiments with similar results. $^{*}P < 0.05$ *vs.* NLE or N. (C) DEPDC1B expression data from TCGA in 90 NSCLC tumor specimens (T) and their paired adjacent non-cancerous lung tissue (N). (D) Representative images of IHC showing the expression of DEPDC1B in normal lung and NSCLC of all clinical stages. Scale bar: 100 μm. (E) DEPDC1B protein expression level in relation to overall survival of 76 NSCLC patients. (F) DEPDC1B expression in relation to overall survival of 464 NSCLC patients (data from TCGA).

3.2. Clinical relevance of DEPDC1B expression level in relation to overall survival of NSCLC patients

The above observed up-regulation of DEPDC1B in NSCLC prompted us to study whether enhanced DEPDC1B expression correlates with tumor progression and prognosis in NSCLC. To this end, Kaplan–Meier survival analysis was performed in clinical specimens from 76 NSCLC patients. As shown in Fig. 1E, patients with high DEPDC1B protein expression had shorter survival (median survival time = 27.8 months) than those expressing low DEPDC1B (median survival time = 39.1 months), In accordance with this finding, Kaplan–Meier survival analysis based on the mRNA expression of DEPDC1B from a large cohort of 464 NSCLC patients provided in the TCGA dataset (http://cancergenome.nih.gov/) also revealed a similar result (Fig. 1F), with a much better prognostic value of DEPDC1B in adenocarcinoma (ADC) than that in squamous cell carcinoma (SCC) (Fig. S1). In order to further evaluate the clinical relevance of DEPDC1B level in relation to overall survival of patients, multivariate analysis with a Cox proportional-hazards model was applied based on the obtained IHC data in 76 NSCLC patients. Our results suggested that DEPDC1B protein level might represent an independent prognostic marker in this cohort of NSCLC ($P = 0.030$, Hazard ratio: 2.060, 95% CI, and 1.073–3.956, Table 1), with patients expressing high levels of DEPDC1B having shorter overall survival time. Consistently, when 248 ADC patients in the TCGA public dataset were analyzed, their DEPDC1B level was also shown to be an independent prognostic factor, displaying a significant inverse correlation with overall survival (Table S2) and positive correlations with the N and T staging of these patients (Table S3). Taken together, data from our own study cohort (Tables 1 and S1) and publically available database (Tables S2 and S3) both support the notion that DEPDC1B might be predictive of the survival of NSCLC patients.

3.3. DEPDC1B promotes migration and invasion of NSCLC cells

In order to understand the biological function of DEPDC1B, A549 and Calu-3 ADC cell lines were used, and stable cell lines overexpressing DEPDC1B (A549/DEPDC1B and Calu-3/DEPDC1B) and vector-control cells (A549/vector and Calu-3/vector) were established (Fig. 2A). We found that overexpression of DEPDC1B led to morphological alterations characteristics of EMT, featured by a scattered distribution of cells with a spindle- or star-like morphology (Fig. S2). Moreover, epithelial markers, namely, E-cadherin, γ-catenin, and CK18, diminished in DEPDC1B-overexpressing cells, while mesenchymal markers such as Vimentin and N-cadherin were dramatically upregulated (Fig. 2B). In subsequent wound healing assays, we found that cells ectopically expressing DEPDC1B migrated more quickly from the wound edge in comparison with the vector control cells (Fig. 2C). In consistence with this result, Transwell migration and matrix penetration assays performed with both NSCLC cell lines showed that DEPDC1B overexpression led to increased cell migration as shown in the Matrigel-free Transwell assay and increased invasion as shown in the Matrigel-coated Transwell assay (Fig. 2D), indicating an enhancing role of DEPDC1B in modulating migration and invasion of NSCLC cells.

3.4. Inhibition of DEPDC1B represses migration and invasion of NSCLC cells

To further study the contribution of endogenous DEPDC1B to migration and invasiveness of NSCLC cells, two human shRNA sequences capable of specifically silencing the expression of DEPDC1B were cloned and transfected in A549 and Calu-3 cells to generate DEPDC1B-knocked down stable cell lines (Fig. 3A), and their effects on EMT as well as cell migration and invasion were evaluated. As expected, knocking down DEPDC1B increased epithelial

Table 1
Univariate and multivariate analyses of various prognostic variables for overall survival in 76 NSCLC patients.

	Univariate analysis		Multivariate analysis	
	P-value	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)
Age				
⩽60	0.017	2.205 (1.149–4.230)	0.230	1.020 (0.988–1.054)
>60				
Sex				
Male	0.578	1.208 (0.620–2.354)		
Female				
Pathologic type				
Squamous cell carcinoma				
Adenocarcinoma	0.463	1.000(0.999–1.002)		
Adenosquamous				
Others NSCLC				
Stage				
I				
II	<0.001	2.837 (1.986–4.054)	<0.001	2.758 (1.947–3.906)
III				
IV				
T				
T1				
T2	0.006	1.686 (1.163–2.443)	0.696	0.917 (0.593–1.418)
T3				
T4				
N				
N0	0.002	2.795 (1.475–5.298)	0.314	0.607 (0.230–1.605)
N1				
M				
M0	<0.001	8.636 (3.443–21.662)	0.487	0.618 (0.159–2.401)
M1				
DEPDC1B				
Low	0.021	2.131 (1.124–4.040)	0.03	2.060 (1.073–3.956)
High				

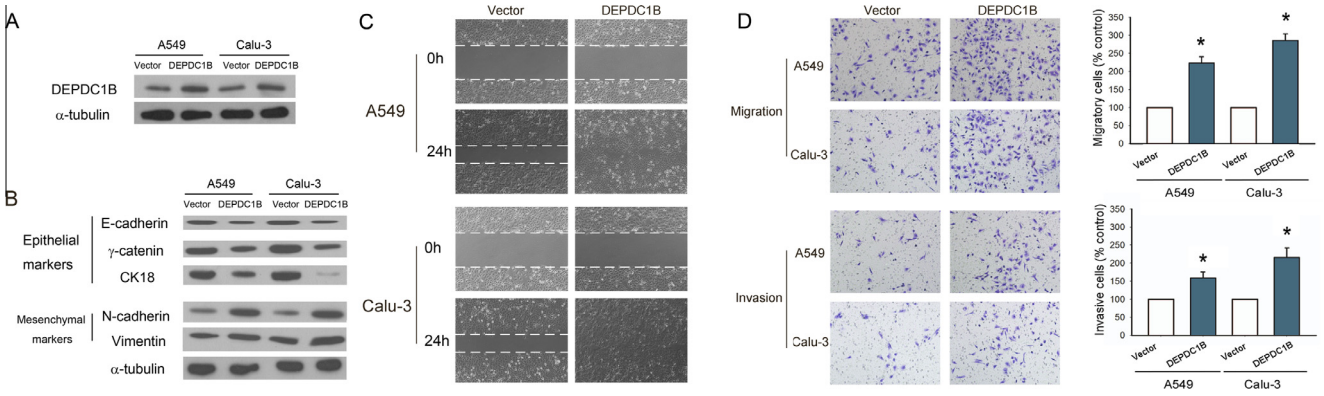

Fig. 2. DEPDC1B promotes migration and invasion of NSCLC cells in vitro. Expression of DEPDC1B (A), as well as epithelial cell markers (E-cadherin, γ-catenin, and CK18) and mesenchymal cell markers (Vimentin and N-cadherin) (B) in indicated cells were examined by WB analysis. (C) Representative images of wound healing assay displaying cell migration to the wound. (D) Representative images of Transwell migration assay (without Matrigel) and Transwell invasion assays (with Matrigel). $^{*}P < 0.05$ *vs.* vector. Error bars represent SEM of three independent experiments.

markers, reduced mesenchymal markers (Fig. 3B), dramatically slowed down wound healing, and attenuated cellular migration and invasion (Fig. 3C and D), supporting the notion that DEPDC1B functions to promote migration and invasion of NSCLC cells.

3.5. DEPDC1B activates Wnt/β-catenin pathway in NSCLC

In the light that the Wnt/β-catenin pathway plays a pivotal role in supporting tumor metastasis, we then assessed the effect of DEPDC1B on Wnt/β-catenin activation. Strikingly, overexpression of DEPDC1B increased the transactivity of β-catenin (Fig. 4A), and subcellular fractionation revealed enhancement of nuclear accumulation of β-catenin in NSCLC cell lines (Fig. 4B), suggesting that DEPDC1B, when expressed at a high level, is capable of activating Wnt/β-catenin signaling. Consistently, ectopic expression of DEPDC1B in A549 and Calu-3 cells markedly induced transcription of Wnt/β-catenin downstream genes, including Axin2, DKK1, MMP7, MMP9 and SOX2 [26–30] (Fig. 4C), further indicating an overactivation of the pathway. In parallel, as shown in Fig. 4A–C, silencing DEPDC1B by siRNA reduced TCF/LEF activities and the nuclear translocation of β-catenin, as well as down-regulated Wnt/β-catenin downstream genes, suggesting a stimulatory role of endogenous DEPDC1B in the regulation of Wnt/β-catenin signaling. Moreover, the potent activation of Wnt/β-catenin by Wnt3a can be remarkably blocked by depletion of DEPDC1B, further providing

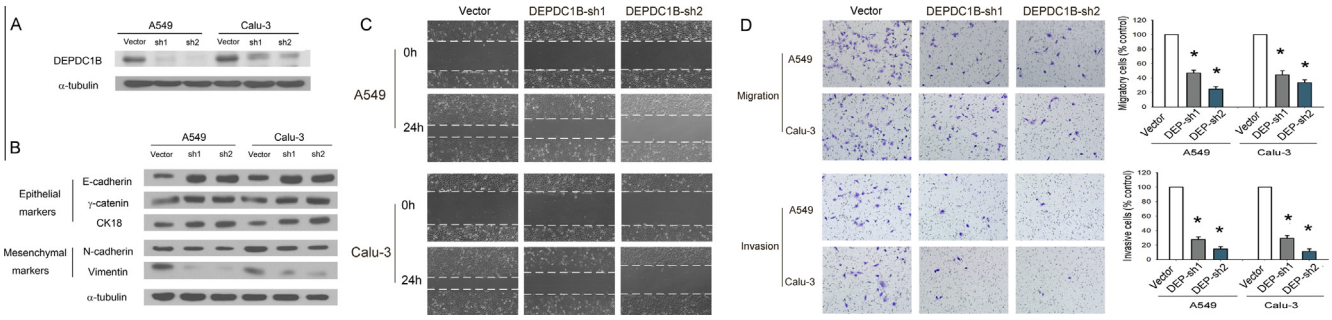

Fig. 3. Inhibition of DEPDC1B represses migration and invasion of NSCLC cells. Expression of DEPDC1B (A), as well as epithelial cell markers (E-cadherin, γ-catenin, and CK18) and mesenchymal cell markers (Vimentin and N-cadherin) (B) in indicated cells were examined by WB analysis. (C) Representative images of wound healing assay displaying cell migration to the wound. (D) Representative images of Transwell migration assay (without Matrigel) and Transwell invasion assay (with Matrigel). $^{*}P < 0.05$ *vs.* vector. Error bars represent SEM of three independent experiments.

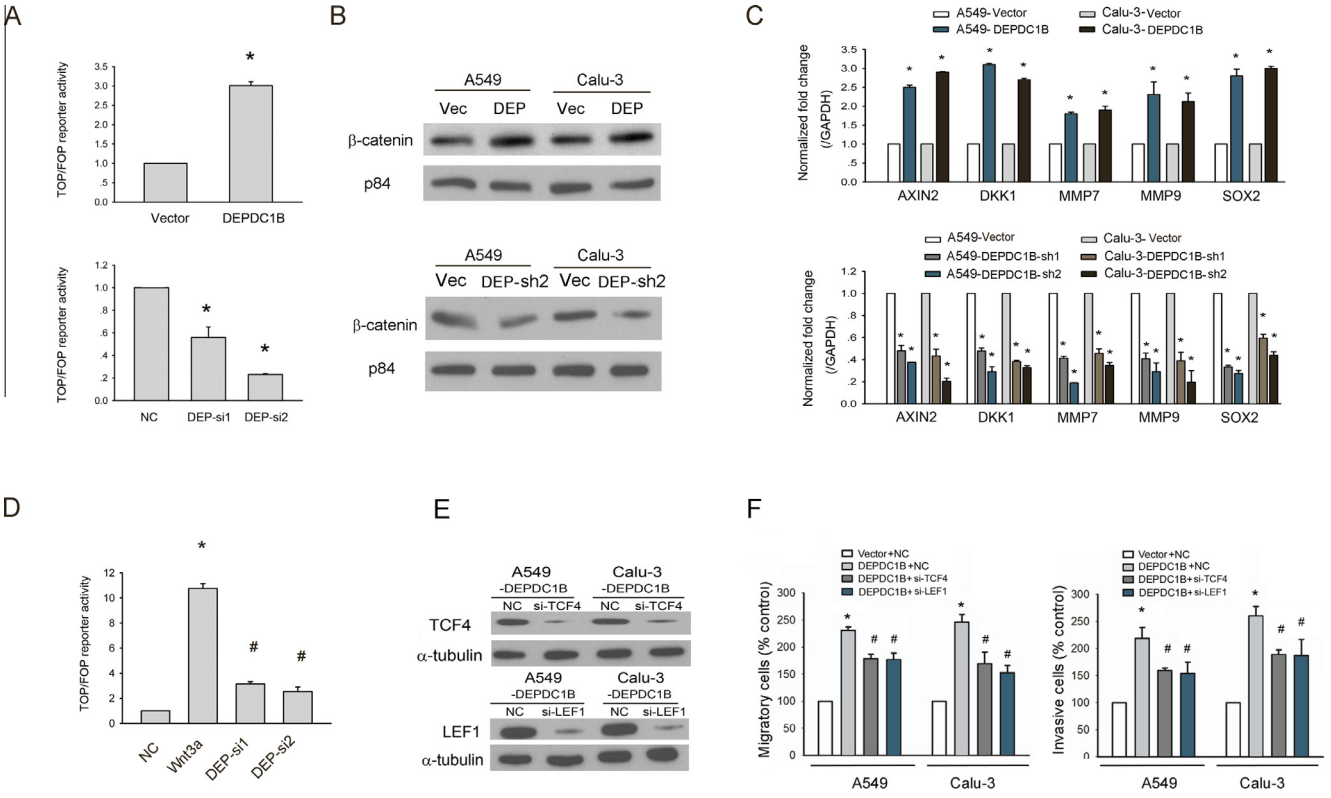

Fig. 4. Activation of Wnt/β-catenin pathway mediates DEPDC1B-enhanced migration and invasion in NSCLC. (A) HEK293 cells transfected with TOPflash or FOPflash and Renilla pRL-TK plasmids were subjected to dual-luciferase assays 48 h after transfection. Reporter activity detected was normalized by Renilla luciferase activity. (B) Nuclear fractions of indicated cells were analyzed by WB analysis with p84 as a loading control. (C) Expression of Wnt/β-catenin downstream target genes was determined by RT-PCR. (D) HEK293 cells were cotransfected with DEPDC1B siRNAs and then treated with Wnt3a for 8 h, followed by dual-luciferase assay. (E) Western blotting analysis confirmed depletion of TCF4 or LEF1 with specific siRNA in DEPDC1B-expressing cells. (F) Quantification of Transwell migration and invasion assays. Error bars represent SEM of three independent experiments. $^{*}P < 0.05$ *vs.* vector (A, C, F) or NC (A, D); $^{\#}P < 0.05$ *vs.* Wnt3a (D) or DEPDC1B (F).

evidence for an important role of DEPDC1B in modulating the signaling.

3.6. DEPDC1B-enhanced migration and invasion in NSCLC is mediated via the Wnt/β-catenin pathway

To validate further the role of Wnt/β-catenin activation in DEPDC1B-promoted cell migration and invasion, we investigated the impact of blocking this pathway by knocking down TCF4 or LEF1 (Fig. 4D) on the invasive capability in DEPDC1B-overexpressing NSCLC cells. As shown in Fig. 4E, inhibition of Wnt/β-catenin signaling partially abrogated DEPDC1B-induced migration and invasiveness. These results indicate that Wnt/β-catenin signaling is a functional mediator of DEPDC1B-induced migration and invasion in NSCLC.

4. Discussion

DEPDC1B is a newly identified gene initially discovered by mRNA expression profiling in MDA-MB 231 human breast cancer cells treated with Raf antisense oligonucleotide, and has been reported to be associated with delayed cell death and increased cell proliferation in breast cancer [31] In this study, using NSCLC as a cancer model, we experimentally found the up-regulation of DEPDC1B in cancer cell lines as well as patient cancer tissue, and that DEPDC1B promotes tumor cell migration and invasion through activating Wnt/β-catenin signaling.

Molecular mechanisms underlying metastasis of NSCLC have been intensively studied. Signaling pathways, including Wnt/β-catenin, Notch, and PI-3K/Akt pathways, have been found to be aberrantly activated and contribute to the metastatic progression of the disease [15,32,33]. In lung cancers, the pro-metastatic role of Wnt/β-catenin signaling has been well documented [14,15]. Mutations of β-catenin, the network hub in this signal pathway, are one of the major causes of aberrant activation in canonic Wnt signaling, and have been frequently reported in several other cancer types, such as colorectal cancer or gastric cancer, whereas mutations of β-catenin are rarely found in NSCLC [16,34,35]. Therefore, identifying factors leading to abnormal activation of Wnt/β-catenin signaling may provide new insights in the abnormality of this important pathway in NSCLC. Our finding that DEPDC1B remarkably increases the transactivity of β-catenin and its downstream genes offers a new mechanism that supports the aberrant activation of Wnt/β-catenin signaling. Importantly, the activation of Wnt/β-catenin is responsible for the DEPDC1B-induced migration and invasiveness, further underscoring the significance of DEPDC1B in NSCLC progression. Although the detailed molecular interactions and cascades that lead to Wnt/β-catenin activation remains to be further investigated, it is of particular note that DEPDC1B contains a DEP domain, which is also a conserved domain of Dishevelled and has been suggested to be essential in mediating the interaction with Frizzled and in activating canonic Wnt/β-catenin signaling. Thus, further determination of whether DEPDC1B also activates the Wnt/β-catenin signaling pathway via its DEP domain might provide new insights in understanding the molecular mechanisms underlying NSCLC metastasis.

Despite our understanding of oncogenic role of DEPDC1B in NSCLC progression, it remains to be clarified how DEPDC1B is up-regulated in NSCLC. It is of particular note that the chromosomal location of DEPDC1B in 5q12, on which phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1) gene also resides, has been previously found to be lack of genomic amplification in NSCLC [36]. Boudreau et al. reported that siRNA knockdown of Raf-1 decreased DEPDC1B protein level, suggesting that its expression might be under the regulation associated with Raf signaling [31]. In addition, employment of a bioinformatic tool (http://ecrbrowser.dcode.org/) also predicts binding of several transcriptional factors, including E2F1, AP2, and ZF5 to the promoter region of DEPDC1B gene. It is therefore of great interest to further study whether these factors may play any roles in upregulating DEPDCB1 in NSCLC. Furthermore, epigenetic mechanisms for the upregulation of DEPDCB1 gene expression, such as promoter/enhancer hypomethylation, should also be investigated.

In summary, we have demonstrated that DEPDC1B is up-regulated in NSCLC and clinically correlated with patient survival in an inverse manner. Our novel finding that the protein contributes to NSCLC cell migration and invasion through activating the Wnt/β-catenin pathway provides a biological clue for future consideration of using this lately identified molecule as a therapeutic target against NSCLC.

Disclosure statement

The authors have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.076.

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