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Original Articles

PIM1 kinase promotes cell proliferation, metastasis and tumor growth of lung adenocarcinoma by potentiating the c-MET signaling pathway

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ABSTRACT

The proto-oncogene PIM1 plays essential roles in proliferation, survival, metastasis and drug resistance in hematopoietic and solid tumors. Although PIM1 has been shown to be associated with lymph node metastasis and poor prognosis in non-small cell lung cancer, its underlying molecular mechanisms in this context are still unclear. Here we show that PIM1 is frequently overexpressed in lung adenocarcinomas, and its expression level is associated with c-MET expression and poor clinical outcome. We further demonstrate that PIM1 may regulate c-MET expression via phosphorylation of eukaryotic translation initiation factor 4B (eIF4B) on S406. Depletion of PIM1 decreased cell proliferation, migration, invasion and colony formation in vitro, as well as reduced tumor growth in vivo. And these effects were partially abrogated by restoring of c-MET expression. Our study implicates a promising therapeutic approach in lung adenocarcinoma patients with PIM1 and c-MET overexpression.

1. Introduction

Lung cancer is the leading cause of cancer-related death worldwide. Lung adenocarcinoma, which accounts for approximately 40% of lung cancer cases, is the most prevalent pathological type of lung cancer [1]. Surgery is currently a viable way to cure lung cancer, but 60–85% of all lung cancer patients are discovered at advanced stages where removal is no longer an option. The low response rate (17–32%) and overall survival (7.4–11.3 months) of platinum-based doublet chemotherapy for patients with advanced lung cancer underscore the critical need to find new actionable targets [2].

Aberrant constitutive activation of tyrosine kinase receptors (RTKs) has been demonstrated in non-small cell lung cancer (NSCLC), especially in lung adenocarcinoma. Subsequent targeted therapies such as epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) inhibitors have been proven effective in multiple clinical trials in patients with EGFR sensitive mutations and ALK fusion genes [3,4]. Although these treatments have been granted FDA approval in first-line NSCLC, emergence of drug resistance remains inevitable. Therefore, it is imperative to further explore new oncogenes and their possible signaling pathways which leading to the development of NSCLC.

PIM1 serine/threonine kinase is encoded by the *Pim-1* proto-oncogene localized on chromosome 6p21.2, and plays a crucial role in cell survival and proliferation [5]. Previous studies have demonstrated that PIM1 is involved in the phosphorylation of a series of cellular substrates, including transcriptional regulators such as MYC, MYB, RuNX1 and RuNX3; cell cycle regulators such as CDKN1A, CDKN1B, CDC25 A/C; cell apoptosis regulators such as BAD, FOXO3a, ASK1; protein translation regulators such as eIF4B and 4EBP1 [6]. Abnormal expression of PIM1 is correlated with the development of various solid and hematopoietic malignancies, including prostate, gastrointestinal, head

Abbreviations: eIF4B, eukaryotic translation initiation factor 4B; RTKs, tyrosine kinase receptors; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; ALK, anaplastic lymphoma kinase; TMA, tissue microarrays; IHC, immunohistochemistry; LN, lymph node

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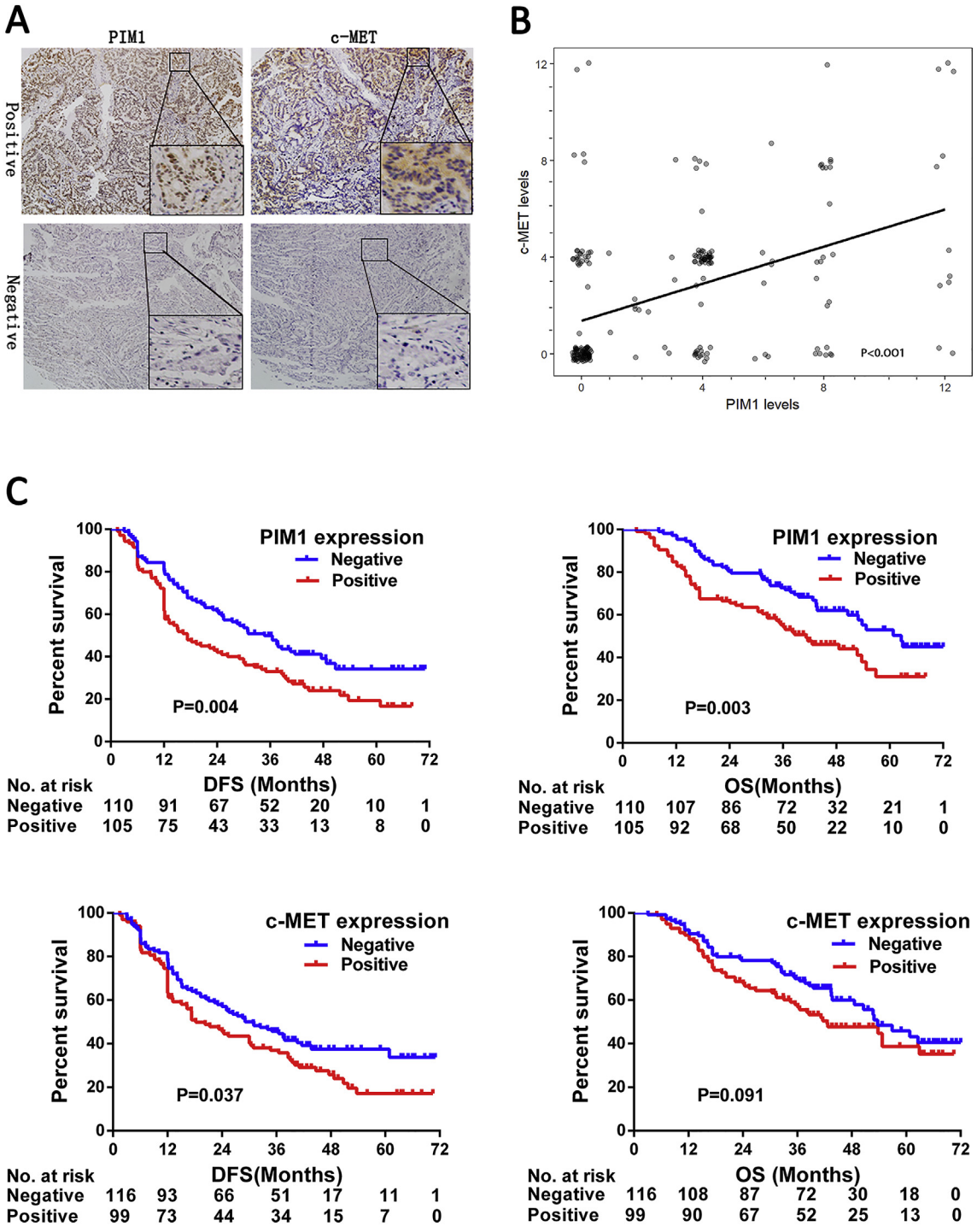

Fig. 1. Analysis of PIM1 and c-MET in lung adenocarcinoma dataset and their correlation in lung adenocarcinoma tissue microarray. (**A**) Images of one same lung adenocarcinoma tissue microarray stained with anti-c-MET and anti-PIM1 antibodies respectively. (**B**) Correlation curve of PIM1 and c-MET expression of lung adenocarcinoma tissue microarray, correlation coefficient (R) and P-value derived by SPSS. (**C**) Kaplan-Meier survival curves of DFS and OS according to PIM1 and c-MET expression (negative vs. positive) in lung adenocarcinoma patients.

and neck and lung cancer [6–8]. Our previous study demonstrated that nuclear PIM1 overexpression associated with lymph node (LN) metastasis, histology and poor survival in NSCLC [9]. Most studies of PIM1 are concentrating on cancers of hematopoietic, prostate or breast origin, yet the mechanisms by which PIM1 promotes cell proliferation, metastasis and tumor growth in lung adenocarcinoma are poorly understood.

PIM1 has been shown to be an important regulator of tyrosine kinase c-MET and its downstream signaling pathways in prostate cancer [10]. Hyperactive c-MET signaling occurs in several types of cancer including NSCLC, where it is overexpressed in 65% of patients [11,12]. Genetic and biochemical data have demonstrated that c-MET, has a causal role in uncontrolled cell growth, survival, metastasis and angiogenesis, thus providing a strong rationale for targeting c-MET in cancer [13–16]. Though no c-MET inhibitors are approved clinically to treat lung adenocarcinoma, several trials have reported efficacy in various cancer types [17–19]. Numerous evidence has shown that c-MET expression can be ascribed to several layers of epigenetic,

Table 1
The association between PIM1 expression and the clinicopathological factors.

Variables	N (%)	PIM1, N (%)		P*
		Negative	Positive	
Age (years)				
< 60	116 (54.0)	54 (46.6)	62 (53.4)	0.143
≥60	99 (46.0)	56 (56.6)	43 (43.4)	
Gender				
Male	100 (46.5)	52 (52.0)	48 (48.0)	0.819
Female	115 (53.5)	58 (50.4)	57 (49.6)	
Smoking Index (SI)				
SI < 400	137 (63.7)	73 (53.3)	64 (46.7)	0.409
SI ≥ 400	78 (36.3)	37 (47.4)	41 (52.6)	
Clinical stage				
I	109 (50.7)	63 (57.8)	46 (42.2)	0.118
II	35 (16.3)	17 (48.6)	18 (51.4)	
IIIA	71 (33.0)	30 (42.3)	41 (57.7)	
Tumor size				
≤3 cm	130 (60.5)	67 (51.5)	63 (48.5)	0.892
> 3 cm	85 (39.5)	43 (50.6)	42 (49.4)	
Regional LN metastasis				
No	115 (53.5)	68 (59.1)	47 (40.9)	**0.012**
Yes	100 (46.5)	42 (42.0)	58 (58.0)	
EGFR mutation				
No	153 (71.2)	76 (49.7)	77 (50.3)	0.492
Yes	62 (28.8)	34 (54.8)	28 (45.2)	
c-MET				
Negative	116 (54.0)	79 (68.1)	37 (31.9)	< 0.001
Positive	99 (46.0)	31 (31.3)	68 (68.7)	

LN: lymph node. AD: adenocarcinoma. SCC: squamous cell carcinoma. SI: smoking index = (number of cigarettes per day) × (duration in years). *P values < 0.05 in bold.

transcriptional and posttranscriptional regulation [20]. Understanding the mechanism by which c-MET is controlled may contribute to better implementation of PIM1-targeting therapeutics and identification of possible therapeutic targets.

In this study, we detected PIM1 and c-MET expression in lung adenocarcinoma by immunohistochemistry and analyzed the association between PIM1 expression and the clinical parameters. Here we demonstrated a positive association between PIM1 and c-MET expression in lung adenocarcinoma tissues, and that high level of PIM1 expression was significantly correlated with disease recurrence and overall survival (OS). Similarly, c-MET expression alone was related with disease recurrence in lung adenocarcinoma patients. Additionally, we explored the mechanism by which PIM1 affects cell proliferation and tumor growth. We elucidated that PIM1 plays a vital part in regulation of c-MET and thus controls cell proliferation, survival, migration and invasion of lung adenocarcinoma cells. Regulation of c-MET in this context is associated with the phosphorylation of eIF4B by PIM1.

2. Materials and methods

2.1. Cell lines and patient samples

Lung cancer cell lines HCC827, PC9, H1975, A549, A549-cis and H1299 were all purchased from National Infrastructure of Cell Line Resource (Beijing, China). Human bronchial epithelial cell BEAS-2B was bought from ATCC and which was isolated from normal human bronchial epithelium obtained from non-cancerous individuals and immortalized by infection with an adenovirus 12-SV40 virus hybrid (Ad12SV40). BEAS-2B was cultured in serum-free LHC-9 medium (Gibco, USA). HCC827, PC9, H1975, A549, A549-cis and H1299 were all cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin and streptomycin (100 units/mL and 100 μg/mL respectively, Life Technologies Inc.). A549-cis referred to A549 cells that were resistant to cisplatin (3 μM) in culture media. All cells were cultured under 5% CO_2 at 37 °C in a humidified incubator.

The retrospective cohort consisted of 215 surgically resected lung adenocarcinoma patients at the Tianjin Cancer Institute & Hospital, Tianjin Medical University, Tianjin P.R. China between 2009 and 2012. Pathologic diagnosis was performed in accordance with the World Health Organization histological classification system and clinicopathologic stage was diagnosed on the basis of the tumor-node-metastasis (TNM) classification system. Prior consent from all patients and approval from the Research Ethics Committee of Tianjin Cancer Institute & Hospital of Tianjin Medical University were obtained for experimentation with human subjects.

2.2. Antibodies and reagents

The antibodies used in this study were listed as follows: anti-PIM1 (#ab1183, Abcam), anti-c-MET (#8198, Cell Signaling Technology), anti-AKT (#9272, Cell Signaling Technology), anti-Phospho-AKT (#4060, Cell Signaling Technology), anti-P44/42 MAPK (#9102, Cell Signaling Technology), anti-Phospho-P44/42 MAPK (#39102, Cell Signaling Technology), anti-Phospho-STAT3 (#9145, Cell Signaling Technology), anti-c-Myc (#9402, Cell Signaling Technology), anti-Phospho-c-Myc (#13748, Cell Signaling Technology), anti-p21 (#2947, Cell Signaling Technology), anti-β-actin (#64132 Bioworld), anti-eIF4B (#3592, Cell Signaling Technology), anti-Phospho-eIF4B (Ser406, #8151, Cell Signaling Technology). The small-molecule inhibitors SGI-1776 and AZD1208 were purchased from Selleck Biochemicals. To treat cells with the inhibitors, according to previously reports [21–25], we set the concentration gradient of 1, 3, 5, 10 μM to SGI-1776 and AZD1208. Cells treated with 10 μM of SGI-1776 and AZD1208 was not used because 60%–70% cell death was observed. And then, Western blot analysis was performed to determine the optimal concentrations. We observed that c-MET was downregulated significantly in A549 and HCC827 cells treated with 5 μM SGI-1776 and 3 μM AZD1208.

2.3. Immunohistochemistry on adenocarcinoma clinical specimens

Immunohistochemistry (IHC) was performed using anti-PIM1 (#ab1183, Abcam) and anti-c-MET (#8198, Cell Signaling Technology) antibodies. The most typical areas of lung adenocarcinoma tissues fixed with formalin and embedded with paraffin were selected to construct tissue microarrays (TMA; 2 mm in diameter). Briefly, xylene and the subsequent gradient ethanol were used to deparaffinize the sections. Antigen unmasking was performed with citrate buffer (pH 6.0) and endogenous peroxidase blocking was performed using 3% hydrogen peroxide. Slides were then washed with Phosphate buffer solution (PBS) and incubated with primary antibodies at 4 °C overnight. To visualize PIM1 and c-MET expression, slides were incubated with ChemMate EnVision Detection Kit (Dako) according to the manufacturer's instructions. Two investigators were blindly examined the slides and reviewed those conflicting ones together to achieve consensus.

Immunoreactivity was semiquantitatively scored according to the estimate of staining intensity and extent of tumor cells. In brief, staining intensity was defined as 0, negative; 1, low; 2, medium; 3, high. While staining extent was defined as 0, 0% of all tumor cells stained; 1, 1%–25% of all tumor cells stained; 2, 26%–50% of all tumor cells stained; 3, 51%–75% of all tumor cells stained; 4, 76%–100% of all tumor cells stained. The final score ranged from 0 to 12 was decided by multiplying the intensity scores with staining extent. The final scores less than or equal than 4 were considered as negative staining while those more than 4 were positive staining.

2.4. Construction of stable cell lines and cell transient transfection

Depletion of PIM1 in HCC827 and A549 cells was achieved using lenti-sgRNA-CAS9 virus followed by selection with puromycin (1 μM). The sgRNAs of PIM1 are as follows: sgRNA1 AGAAGGACCGGATTTCC GAC; sgRNA2 ACCATCGAAGTCCGTGTAGA; sgRNA3 ATCTCTTCGTC

Table 2
Univariate and multivariate analyses of DFS and OS in all patients.

Variables	DFS					OS				
	Univariate Analysis			Multivariate Analysis		Univariate Analysis			Multivariate Analysis	
	5-year DFS rate (%)	HR (95%CI)	P*	HR (95%CI)	P*	5-year OS rate (%)	HR (95%CI)	P*	HR (95%CI)	P*
All patients (n = 215)										
Age (years)										
< 60	34.5	0.983 (0.708–1.365)	0.921			53.4	1.049 (0.710–1.548)	0.811		
≥60	32.3					54.5				
Gender										
Male	33.0	1.050 (0.757–1.458)	0.769			33.0	1.188 (0.806–1.752)	0.385		
Female	33.9					33.9				
Smoking Index (SI)										
SI < 400	35.8	1.438 (1.027–2.014)	**0.034**	1.506 (1.068–2.124)	**0.019**	54.0	1.340 (0.891–2.017)	0.160		
SI ≥ 400	29.5					53.8				
Clinical stage										
I- II	39.4	1.826 (1.302–2.561)	< **0.001**	1.681 (1.190–2.374)	**0.003**	61.3	1.756 (1.173–2.628)	**0.006**	2.046 (1.341–3.120)	**0.001**
IIIA	18.8					40.6				
Tumor size										
≤3 cm	33.8	0.979 (0.702–1.367)	0.902			56.2	0.570 (0.757–1.658)	0.570		
> 3 cm	32.9					50.6				
Regional LN metastasis										
No	38.3	1.393 (1.005–1.932)	**0.047**			60.9	1.417 (0.960–2.093)	0.080		
Yes	28.0					46.0				
Adjuvant treatment										
No	33.1	0.979 (0.691–1.388)	0.907			49.7	0.612 (0.394–0.950)	**0.029**	0.448 (0.280–0.719)	**0.001**
Yes	34.3					62.9				
EGFR mutation										
No	33.3	0.866 (0.604–1.241)	0.433			54.2	0.792 (0.516–1.214)	0.284		
Yes	33.9					53.2				
PIM-1										
Negative	40.9	1.613 (1.160–2.241)	**0.004**	1.389 (0.988–1.953)	**0.059**	62.7	1.806 (1.219–2.676)	**0.003**	1.674 (1.110–2.525)	**0.014**
Positive	25.7					44.8				
MET										
Negative	40.5	1.416 (1.021–1.964)	**0.037**			59.5	1.398 (0.948–2.062)	0.091		
Positive	25.3					47.5				

DFS: disease-free survival. OS: overall survival. HR: hazard ratio. CI: confidence interval. LN: lymph node. AD: adenocarcinoma. SCC: squamous cell carcinoma. SI: smoking index = (number of cigarettes per day) × (duration in years). *P values < 0.05 in bold.

ATGCTCGAA; Control sgRNA CGCTTCCGCGGCCCGTTCAA (Fig. 2C). The lenti-sgRNA-CAS9 virus was constructed by Genechem. After infection with lenti-sgRNA-CAS9 virus, pooled cells were used for subsequent experiment. The c-MET plasmid was purchased from Addgene to restore c-MET expression in PIM1-knockout HCC827 and A549 cells. BEAS-2B and H1299 cells with enforced expression of PIM1 were generated by transfecting cells with the PIM1 expression vector purchased from Genechem. To generate this expression vector, the full-length human PIM1 cDNA (Genbank Accession number NM_002648) was amplified by PCR with primers 5′- GAGGATCCCCGGGTACCGGTCGCC ACCATGCTCTTGTCCAAAATCAACTC -3′ and 5′- TCCTTGTAGTCCATA CCTTTGCTGGGCCCCGGCGACAGG -3'. The PCR products were subcloned into the GV365 vector (Genechem, Shanghai, China) by restriction digestion with AgeI. And the PIM1 construct was confirmed by sequencing. Knockdown of PIM1 was performed using SMARTpool siRNAs (Dharmacon RNAi Technologies) and a non-targeting siRNA served as negative control. The transfection of plasmids and siRNA were performed with Lipofectamine® 3000 reagent (Thermo Scientific) according to the manufacturer's instructions.

2.5. Western blot analysis

Whole cell extracts were made using RIPA lysis buffer containing proteinase inhibitor cocktail (Roche). After centrifugation, lysates were then quantified with the Pierce BCA Protein Assay Kit (Thermo). To analyze the protein expression level, 20 μg cell extract of each sample was denatured and separated by 10% sodium dodecyl sulfate-polyacrylamide gels. A polyvinylidene fluoride (PVDF) membrane was used to transfer the protein extracts after electrophoresis. PVDF membranes were then incubated with primary antibodies overnight at 4 °C. Anti-rabbit-HRP (ZSGB-BIO) secondary antibody was incubated for 90 min at room temperature the following day. After washing, specific proteins were detected using Western Lightning Plus-ECL (Millipore Corporation, Billerica, U.S.A).

2.6. Cell growth and viability assays

After lentiviral infection or plasmid transfection, cells were then cultivated in 96-well plates at a density of 2500–3000 cells per well. Cell viability was accessed at 24, 48, 72 and 96 h using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) according to the manufacturer's protocol and absorbance was quantified at 490 nm by Gen5 data analysis software. Each assay included 6 replicate wells and was repeated three times. For colony forming assays, 500 cells were seeded and cultured in 6-well plates for 14 days, then fixed with 100% methanol and dyed with crystal violet. Colonies (> 50 cells) were subsequently counted manually.

2.7. Wound-healing assay

For wound-healing assays, cells (6–8 × 10^5 cells) were seeded and cultured to near confluency in 6-well plates. Three parallel wounds were made using 20 μl sterile pipette tips. Cells were then washed with PBS and cultured with RPMI-1640 medium containing 1% FBS. Images were taken at 0, 12, 24, 36 and 48 h after scratching.

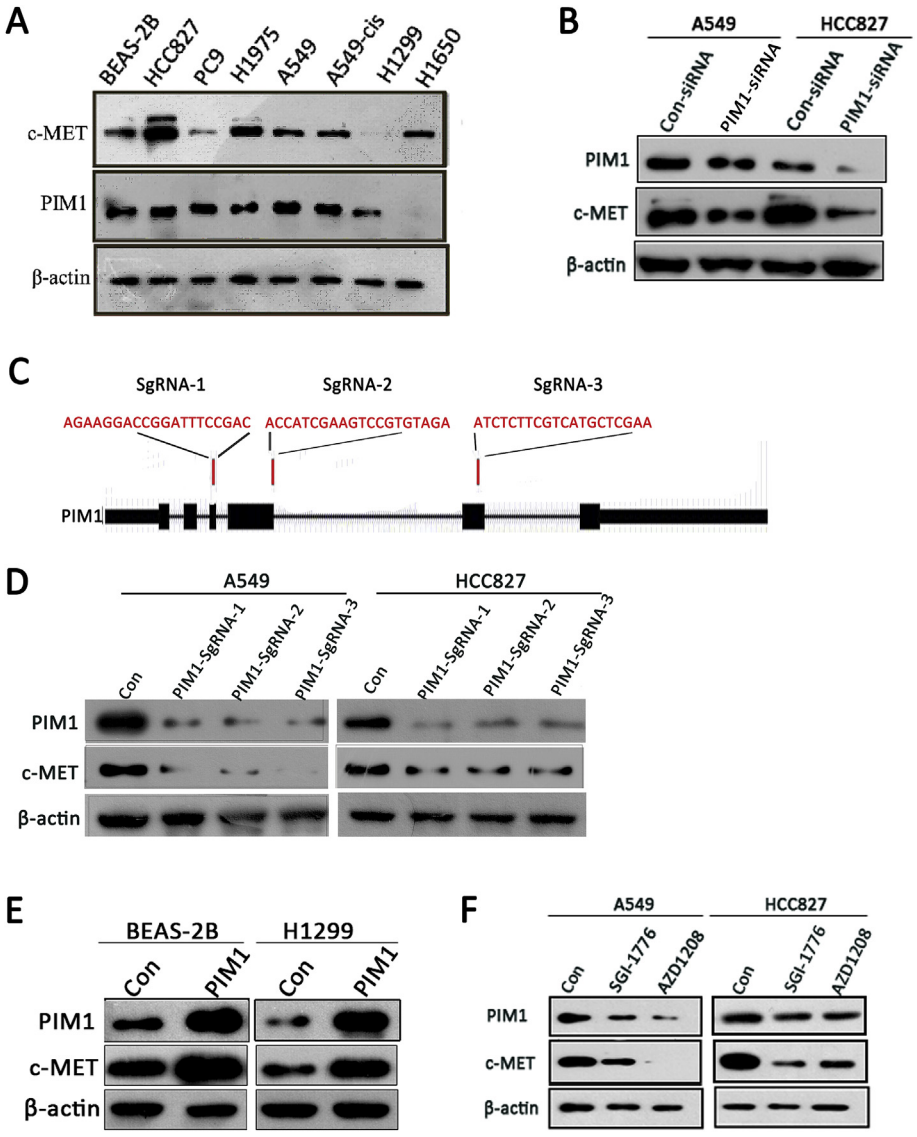

Fig. 2. PIM1 regulates c-MET expression in lung adenocarcinoma cell lines. (**A**) PIM1 and c-MET expression of human bronchial epithelial cell BEAS-2B and a panel of lung adenocarcinoma cell lines analyzed by Western blot. (B) Effects of treatment with a non-target control siRNA or PIM1 siRNA for 96 h on c-MET expression in A549 and HCC827 cells analyzed by Western blot. (**C**) Image of the regions of three targeted sgRNAs constructed in the CRISPR/CAS9 lentivirus in PIM1 gene. (**D**) Effects of treatment with a lentivirus expressing Cas9 and a control sgRNA or sgRNAs targeting PIM1 on c-MET expression in A549 and HCC827 cells analyzed by Western blot. (**E**) Effects of treatment with an unloaded control or a plasmid expressing PIM1 on c-MET expression in BEAS-2B and H1299 cells. (**F**) Effects of treatment with two PIM1 inhibitors SGI-1776 (5 μM) and AZD1208 (3 μM) for 24 h on c-MET expression in A549 and HCC827 cells analyzed by western blot.

2.8. Transwell assay

Transwell chambers with or without Matrigel were used to perform cell invasion or migration assays. In brief, cells (3×10^4 cells) re-suspended in 200 μl serum-free RPMI-1640 medium were added to the upper chamber of each 24-well culture inserts and 500 μl RPMI-1640 supplemented with 20% FBS was placed to the lower chamber. Cells were allowed to migrate for 24 (A549) or 36 h (HCC827) and invade for 24 (A549) or 48 h (HCC827). Cells remained on the upper surface of the chamber were cleaned, whereas cells passed through the membrane were fixed in 4% paraformaldehyde and stained with crystal violet. The migrated or invaded cells were counted and imaged.

2.9. In vivo assay

Five-week-old NUDE mice (Nanjing Biomedical Research Institute of Nanjing University) were acclimated in a specific pathogen free animal facility at least 1 week before use. Mice were randomly divided into three groups. Group one was injected with HCC827 cells infected with negative control lentiviral, group two was injected with HCC827 cells infected with lenti-sgRNA-CAS9 virus targeting PIM1 and group three was injected with HCC827 cells infected lenti-sgRNA-CAS9 virus targeting PIM1 as well as transfection with c-MET plasmid. Cells were inoculated subcutaneously into the leg region with 5×10^6 cells in 100 μl PBS per injection. Measurements of the maximum perpendicular tumor diameters were performed by vernier calipers, and tumor volume was accessed using the formula of $\pi \times$ (width) $^2 \times$ length/6. Significant differences between the control versus the treated groups were determined using ANOVA. Prior consent was obtained from the Animal Care and Use Committee of Tianjin Cancer Institute & Hospital of Tianjin Medical University for animal experiments.

2.10. Statistical analysis

Chi-squared test or Fisher exact test was performed to analyze the relationship between PIM1 and clinicopathologic parameters. Cox proportional hazards regression with the backward elimination method was used to evaluate the hazard ratios for those positive risk factors. Spearman correlation analysis was used to analyze the correlation between c-MET and PIM1 expression level. The statistical significance of other experimental results was accessed using Student's unpaired *t*-test, and data were shown as mean ± standard deviation (SD). A two-sided p-value of < 0.05 was adopted to be statistically significant.

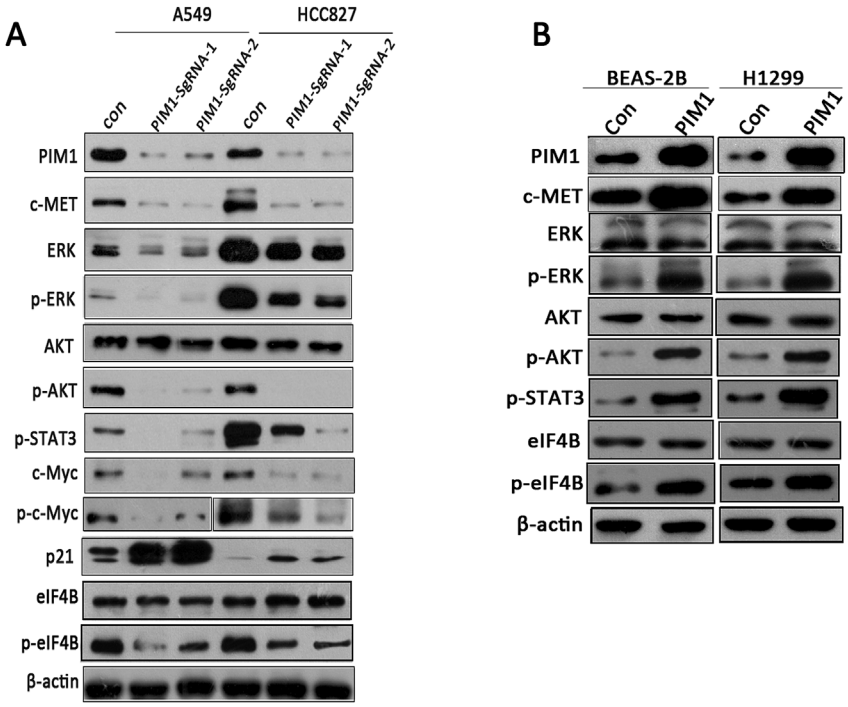

Fig. 3. PIM1 regulates the expression of c-MET and its downstream effectors via phosphorylation of eIF4B. (**A**) Effects of treatment with a lentivirus expressing Cas9 and a control sgRNA or sgRNAs targeting PIM1 on the indicated proteins analyzed by Western blot. (**B**) Effects of treatment with an unloaded control or a plasmid expressing PIM1 on the indicated proteins in BEAS-2B and H1299 cells analyzed by Western blot.

3. Results

3.1. PIM1 expression was positively associated with c-MET expression and poor clinical outcome in lung adenocarcinoma patients

To identify the relevance of PIM1 and c-MET proteins and their prognostic value in lung adenocarcinoma, we performed immunohistochemical staining of PIM1 and c-MET on 215 resected lung adenocarcinomas. PIM1 protein predominantly detected at cell nucleus was highly expressed in 44.8% of lung adenocarcinoma cases while c-MET exhibited membrane staining was overexpressed in 51.2% of lung adenocarcinoma cases (Fig. 1A). Expression levels of PIM1 and c-MET were positively correlated (correlation coefficient [R] = 0.4375, $P < 0.001$) (Fig. 1B). Univariate survival analysis indicated that high level of PIM1 expression was correlated with disease recurrence and overall survival (OS) while c-MET expression was related only to disease recurrence (Fig. 1C). Further multivariate survival analysis demonstrated that PIM1 was an independent prognostic marker (Table 2) and correlated with lymph node metastasis suggesting a close relationship between PIM1 overexpression and disease progression (Table 1).

3.2. PIM1 kinase regulates c-MET protein in lung adenocarcinoma cells

To examine the relationship between PIM1 and c-MET expression in lung adenocarcinoma, human bronchial epithelial cell and 7 lung adenocarcinoma cells were used to detect protein expression. c-MET protein was detectable by western blot in 7 of the 8 cell lines all of which also expressed PIM1 (Fig. 2A). To determine whether PIM1 kinase has a causal role on c-MET expression, we applied several different methods. RNA interference-mediated silencing of PIM1 expression in HCC827 and A549 cells with relatively high expression of PIM1 resulted in markedly reduced expression of c-MET (Fig. 2B). Additionally, we used a lentivirus expressing the Cas9 nuclease and a control single-guide RNA (SgRNA) or one of three sgRNAs directed against PIM1 (Fig. 2C) to infect HCC827 and A549 cells. Western blotting demonstrated a near-complete loss of PIM1 with three SgRNAs accompanied by a marked downregulation of c-MET levels (Fig. 2D). Similarly, ectopic expression of PIM1 in BEAS-2B and H1299 with relatively low PIM1 expression resulted in increased levels of c-MET (Fig. 2E). We further found that PIM1 induced c-MET expression required PIM1 kinase activity since c-MET expression was significantly reduced when HCC827 and A549 cells were co-cultured with small-molecule PIM inhibitors (Fig. 2F).

3.3. PIM1 potentiates the c-MET signaling pathway might via phosphorylation of eIF4B on S406

c-MET is known to have three main downstream effector components including MAPK, STAT3 and PI3K-AKT cascades. Since our above results have demonstrated that PIM1 could regulate c-MET expression, we sought to determine whether PIM1 plays a key role in activating c-MET downstream signaling pathways. As expected, when PIM1 was knockout by CRISP/Cas9 nuclease system, in addition to the classical downstream effectors like c-Myc and p21, c-MET and its downstream effectors including activated ERK, STAT3 and AKT were all dramatically downregulated (Fig. 3A). Similarly, overexpression of PIM1 in BEAS-2B and H1299 cells resulted in enhanced activation of ERK, STAT3 and AKT (Fig. 3B). These results demonstrate thoroughly that PIM1 plays an important part in potentiating c-MET downstream signaling pathway.

To date, four main mechanisms have been found to explain the overactivation of the c-MET, including point mutations, increased transcription of the *met* gene, copy number alterations and translational regulation [26–29]. The phosphorylation of eIF4B was reported to have the capacity to facilitate the binding of the c-MET and eukaryotic initiation factor 3 (eIF3) complex, thus enhancing the expression of c-MET [30]. Cen et al. demonstrated that PIM1 manipulates the translation of c-MET expression by phosphorylation of eIF4B S406 in various malignant tumors [10]. To identify the mechanism by which PIM1 controls c-MET expression and its downstream signaling pathway, we assessed the total and S406 phosphorylation status of eIF4B. Consistent with Cen et al.'s findings, the phosphorylation of eIF4B on S406 changed concomitant with the downregulation or upregulation of PIM1 and c-MET levels (Fig. 3A and B). This analysis suggested that PIM1 potentiates the c-MET signaling pathway in lung adenocarcinoma might partially via phosphorylation of eIF4B on S406.

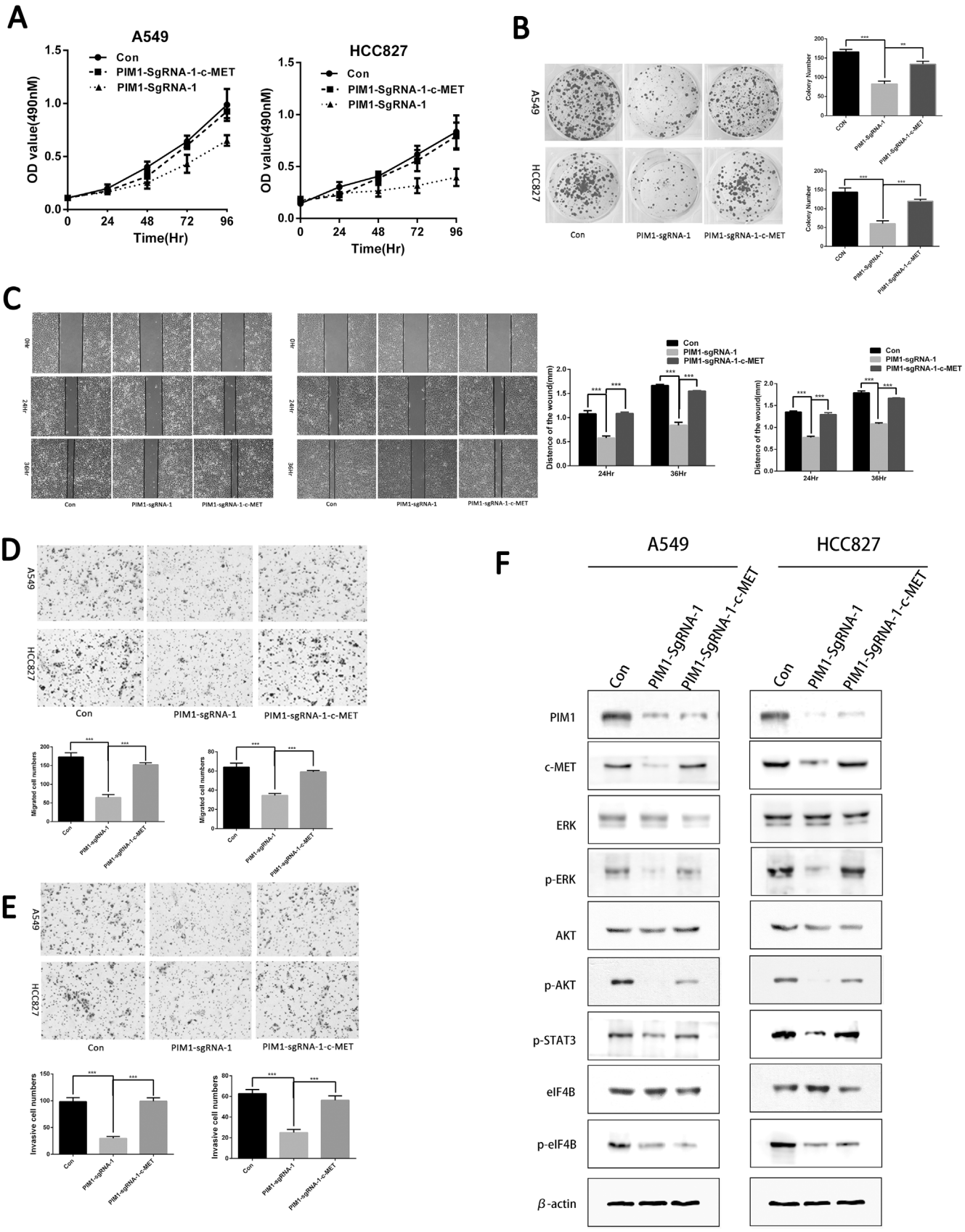

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Fig. 4. PIM1/c-MET signaling pathway regulates cell proliferation, survival, invasion and metastasis in vitro. Experiments in vitro were all divided into three groups: cells infected with negative control lentiviral, cells infected with lenti-sgRNA-CAS9 virus targeting PIM1 and cells infected lenti-sgRNA-CAS9 virus targeting PIM1 as well as transfection with c-MET plasmid. **(A)** Cell growth curves of the indicated groups by MTT assay. Data shown are representative of three independent experiments. (**B**) Colony formation of the indicated groups and their associated quantification. Data shown are representative of three independent experiments presented as mean ± s.e.m. ** P < 0.01, *** P < 0.001. (**C**) Cell migration of the indicated groups by wound healing assay and their associated quantification. Data shown are representative of three independent experiments presented as mean ± s.e.m. *** P < 0.001. (**D**) Cell migration through a transwell chamber of the indicated groups and their associated quantification. Data shown are representative of three independent experiments presented as mean ± s.e.m. *** P < 0.001. (**E**) Cell invasion through a transwell chamber coated with Matrigel of the indicated groups and their associated quantification. Data shown are representative of three independent experiments presented as mean ± s.e.m. *** P < 0.001. (**F**) Western blot validation of lysates from A549 and HCC827 cells after PIM1-SgRNA-1 infection and c-MET transfection.

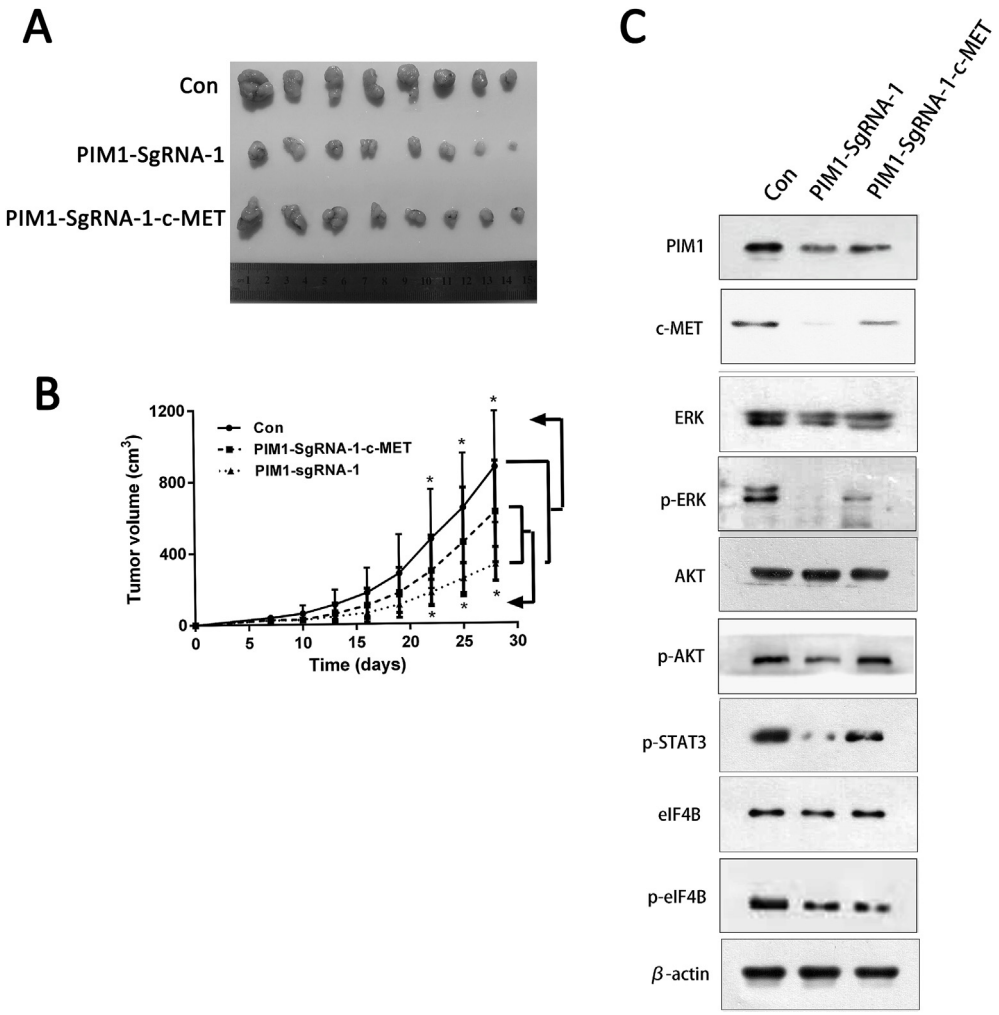

Fig. 5. PIM1/c-MET signaling pathway regulates tumor growth in vivo. Groups of experiments in vivo were the same as that in vitro. (**A**) In vivo tumor growth in nude mice of the indicated groups (n = 8 tumors per group). (**B**) Quantification of tumor volume over time. * P < 0.05. (**C**) Western blot validation of lysates from xenograft tumors of three groups.

3.4. PIM1/c-MET signaling pathway regulates cell proliferation, survival, invasion and metastasis in vitro and tumor growth in vivo

c-MET is discovered to have important functions in cell growth, survival and metastasis of multiple human cancer types via its downstream effectors. The ability of PIM1 to regulate c-MET expression suggested the possibility that this pathway may play a vital function in lung adenocarcinoma. In addition to PIM1 knockout A549 and HCC827 cells, we restored c-MET expression in A549 and HCC827 cells in which PIM1 was knockout by CRISP/Cas9 nuclease system to further investigate whether PIM1 indeed functioned through c-MET (Fig. 4F). Knockout of PIM1 decreased A549 and HCC827 cell proliferation compared to control cell lines (Fig. 4A). Consistently, knockout of PIM1 prevented colony formation of A549 and HCC827 cells (Fig. 4B). Furthermore, the in vitro migration and invasive capacity of PIM1 knockout A549 and HCC827 cells was significantly reduced (Fig. 4C–E). As expected, when c-MET expression was restored in PIM1 knockout A549 and HCC827 cells, the PIM1-induced effects on proliferation, survival, migration and invasion were partially reversed (Fig. 4A–E). To further investigate whether PIM1 indeed functioned through c-MET, we examined the expression of ERK, p-ERK, AKT, p-AKT, p-STAT3 in cells with PIM1-sgRNA-c-MET, western blot analysis showed that p-ERK, p-AKT, p-STAT3 are all restored when c-MET is overexpressed in A549 PIM1-SgRNA-1 and HCC827 PIM1-SgRNA-1 cells (Fig. 4F).

We then examined whether PIM1/c-MET signaling pathway functioned in vivo. Nude mice injected subcutaneously with HCC827 cells either infected with negative control lentivirus, lenti-sgRNA-CAS9 virus targeting PIM1, or lenti-sgRNA-CAS9 virus targeting PIM1 plus c-MET

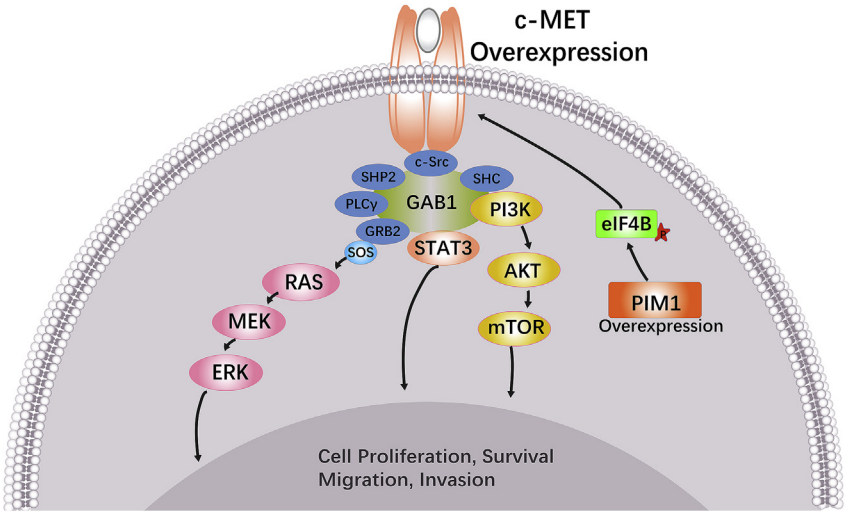

Fig. 6. Model depicting role of PIM1 in regulating the expression of c-MET signaling. Overexpression of PIM1 in lung adenocarcinoma induces eIF4B phosphorylation. Phosphorylated eIF4B further accelerates c-MET mRNA translation, thereby potentiating RAS/ERK, PI3K/AKT and STAT3 pathways in cooperation with oncogenic drivers leading to increased cell proliferation, survival, migration and invasion.

transfection. Compared to control group, all eight mice injected with PIM1 knockout HCC827 cells resulted in significantly reduced tumor growth. However, all eight mice injected with PIM1 knockout HCC827 cells ectopically expressing c-MET grew large tumors (Fig. 5A and B). Xenograft tumors were then used to perform western blot analysis. Consistent with the results in vitro, PIM1 expression was nearly absent and c-MET level was significantly downregulated in PIM1 knockout xenograft tumors. In addition, c-MET expression along with its downstream effectors p-ERK, p-AKT, p-STAT3 were all restored in PIM1 knockout xenograft tumors with c-MET overexpression ectopically. (Fig. 5C). These findings indicate that PIM1/c-MET signaling pathway plays an important part both in vitro and in vivo.

4. Discussion

PIM1 has a causal role in uncontrolled cell growth, survival and metastasis and has been validated as a hopeful therapeutic target in various malignant tumors. However, the molecular mechanism by which PIM1 functioned in lung cancer remains unclear. Understanding the specific molecules PIM1 regulated in lung adenocarcinoma is of great significance to discover new effective anticancer therapeutic strategy. This study uncovered the positive association between PIM1 and c-MET expression in lung adenocarcinoma tissues and further proposed a model that PIM1 served as a valid manipulator of c-MET as well as its downstream signaling pathways including RAS/ERK, PI3K/AKT and STAT3 (Fig. 6). Moreover, our data confirmed the function of PIM1/c-MET signaling pathway in proliferation, survival, metastasis and invasion of lung adenocarcinoma cells. These findings uncover a new molecular mechanism of PIM1 in the context of lung adenocarcinoma.

Relationship between PIM1 expression and prognosis has been analyzed in various cancer types, however the results have varied. Studies on hematological malignancies [31], gastric cancer [32] and head and neck cancer [33] have demonstrated that aberrant expression of PIM1 was associated with unfavorable survival, while it appeared to be a favorable prognostic factor in pancreatic cancer [34]. Our previous study showed that nuclear PIM1 overexpression associated with LN metastasis, histology and poor clinical outcome in both lung adenocarcinoma and squamous cell carcinoma [9]. Consistent with our previous study, here we identified PIM1 as an independent poor prognostic factor for lung adenocarcinoma which suggests that PIM1 may serve as a promising therapeutic target.

EIF4B is one of the eukaryotic translation initiation factors which controls protein translation [35]. Previous studies have explored the relationship between PIM1 and eIF4B. Yang et al. identified that eIF4B is an important substrate of PIM1 kinase [36] and Zemskova et al. reported that PIM1 expression in prostate fibroblasts could phosphorylate 4EBP1 and eIF4B which regulate 5′Cap driven protein translation [37]. Various mechanisms have all been confirmed to play essential roles governing the activity of c-MET, including DNA methylation [38], copy number alterations, point mutations, increased transcription of the *met* gene [39,40], glycosylation [41], phosphorylation [42], internalization and degradation [43,44] and translational regulation. Cen et al. demonstrated that PIM1 phosphorylates eIF4B S406 and thereby manipulating the translation of c-MET expression in prostate tissues and leukemic cells from AML patients as well as cell lines representing various malignant tumors [10]. In our study, we discovered that PIM1 played a vital role in the regulation of c-MET in lung adenocarcinoma, and the phosphorylation of eIF4B on S406 changed concomitant with the alteration of PIM1 and c-MET levels. With the limited evidence, we considered that this regulation of c-MET by PIM1 might partially via eIF4B phosphorylation on S406.

Previous studies have identified that upregulated PIM1 kinases has a positive role in promoting cell motility through various mechanisms [45]. Our study observed that downregulation of PIM1 had a negative effect on tumor cell proliferation, survival, migration and invasion, however those negative effects were significantly abrogated when c-MET was restored. The same phenomena were also observed in vivo. The physiological relevance may be associated with the regulation of PIM1 on c-MET downstream signaling pathways including RAS/ERK, PI3K/AKT and STAT3 pathways. Activated ERK can translocate to the nucleus and activate a series of transcription factors that subsequently upregulate a large number of genes involved in cell proliferation, motility and cell cycle progression [46]. PI3K binding to c-MET and the subsequent activation of AKT is responsible for cell survival [47]. Direct binding of STAT3 to c-MET leads to its translocation to nucleus and results in tubulogenesis and invasion [48]. In lung adenocarcinoma patients, we also observed that high level of PIM1 correlated with lymph node metastasis and high expression of PIM1 and c-MET as well as related to poor outcome. Our results suggest that PIM1 inhibitors or the combination of PIM1 and c-MET inhibitors may have therapeutic relevance in lung adenocarcinoma.

Except for c-MET along with its downstream effectors, we also detected p-c-Myc and p21, known as cooperator and substrate of PIM1. PIM1 in cooperation with c-Myc could enhance tumor cell growth and PIM1 phosphorylated cell cycle regulators p21 could leading to cell cycle progression [49–51]. In our study, restoring of c-MET in PIM1 knockout cells could only partially restore the cell proliferation in vivo or in vitro, suggesting that other signaling pathways, such as PIM1-mediated phosphorylation of p-c-Myc and p21, also play critical roles

on lung adenocarcinoma cell proliferation and survival.

Our data show that PIM1 expression not only positively associates with c-MET expression in lung adenocarcinoma tissues but also plays a vital part in the expression of c-MET as well as its downstream signaling pathways including RAS/ERK, PI3K/AKT and STAT3. This manipulation is achieved by the phosphorylation of eIF4B. This observation provides a new molecular mechanism of PIM1 in lung adenocarcinoma. Moreover, our data confirmed that PIM1/c-MET signaling pathway functions in proliferation, survival, metastasis and invasion of lung adenocarcinoma cells. Based on our work, we propose that inhibition of PIM1/c-MET signaling may be promising therapeutic target in appropriately selected lung adenocarcinoma patients with overexpression of PIM1 and c-MET.

Conflicts of interest

The authors declare no potential conflicts of interest.

Acknowledgments

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