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The histone demethylase PHF8 is an oncogenic protein in human non-small cell lung cancer

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ABSTRACT

PHF8 is a JmjC domain-containing protein and erases repressive histone marks including H4K20me1 and H3K9me1/2. It binds to H3K4me3, an active histone mark usually located at transcription start sites (TSSs), through its plant homeo-domain, and is thus recruited and enriched in gene promoters. PHF8 is involved in the development of several types of cancer, including leukemia, prostate cancer, and esophageal squamous cell carcinoma. Herein we report that PHF8 is an oncogenic protein in human non-small cell lung cancer (NSCLC). PHF8 is up-regulated in human NSCLC tissues, and high PHF8 expression predicts poor survival. Our *in vitro* and *in vivo* evidence demonstrate that PHF8 regulates lung cancer cell proliferation and cellular transformation. We found that PHF8 knockdown induces DNA damage and apoptosis in lung cancer cells. PHF8 promotes miR-21 expression in human lung cancer, and miR-21 knockdown blocks the effects of PHF8 on proliferation and apoptosis of lung cancer cells. In summary, PHF8 promotes lung cancer cell growth and survival by regulating miR-21.

1. Introduction

Chromatin dynamics are affected by histone modifications. Chromatin regulators can alter histone modifications and thus are extensively linked to gene activation and repression [1]. Plant Homeo Domain Finger protein 8 (PHF8) is a JmjC domain-containing protein and erases repressive histone marks including H4K20me1 and H3K9me1/2 [2–4]. PHF8 binds to H3K4me3, an active histone mark usually located at transcription start sites (TSSs), through its plant homeo-domain (PHD), and is thus recruited and enriched in gene promoters [5,6]. Chromatin immunoprecipitation-sequencing (ChIP-seq) data from immortalized human HeLa cells show that about 72% of PHF8 binding sites are at promoters [3]. Also, PHF8 regulates the cell cycle biological process *via* removing H4K20me1 from the promoters of certain E2F1-regulated genes [2]. Interestingly, altering PHF8 levels in HeLa cells affects H4K20me1 methylation only in late G2/M and early G1 stages of the cell cycle, not globally [2]. PHF8 also binds to rRNA gene promoters and demethylates H3K9me2/1 to activate rRNA synthesis [4,7], functioning as an activator [8].

PHF8 functions essentially in various development and disease processes. Mutations in PHF8 are associated with X linked mental retardation and cleft lip/cleft palate [8–11]. Histone methylation modulated by PHF8 plays a critical role in neuronal differentiation, brain and craniofacial development [2,12]. Recently, PHF8 was reported to participate in several types of cancer, including prostate cancer, leukemia and esophageal squamous cell carcinoma. Björkman et al. [13] carried out a systematic, genome-wide analysis of the functional significance of 615 epigenetic proteins in prostate cancer (PrCa) cells. They identified that PHF8 was overexpressed in prostate cancer with an impact on cell proliferation, migration and invasion [13]. In acute promyelocytic leukemia, PHF8 governs retinoic acid response. *All-trans* retinoic acid (ATRA) sensitivity depends on the enzymatic activity and phosphorylation status of PHF8, which can be pharmacologically manipulated to resurrect ATRA sensitivity to resistant cells [14]. Oncogenic features of PHF8 in esophageal squamous cell carcinoma (ESCC) was also reported [15].

Here in this study, we report PHF8 as an oncogenic protein in human non-small cell lung cancer (NSCLC). PHF8 overexpresses in human NSCLC tissues and predicts poor survival. We demonstrate that PHF8 regulates lung cancer cell proliferation and cellular transformation, and PHF8 knockdown induces DNA damage and apoptosis in lung cancer cells. Furthermore, PHF8 regulates miR-21 expression in human lung cancer, and miR-21 knockdown blocks the effects of PHF8 on proliferation and apoptosis of lung cancer cells.

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2. Materials and methods

2.1. Patients

60 patients with NSCLC were enrolled in this study. The patients were recruited at Shanghai Chest Hospital, Shanghai Jiao Tong University (Shanghai, China). The 17 adjacent normal tissues were obtained from NSCLC patients undergoing surgery. The clinical characteristics of the patients is listed in Table 1. The diagnosis of lung cancer was established using World Health Organization (WHO) morphological criteria. Clinicopathological parameters including age, gender, smoking history, histologic grade, tumor status, lymph node metastasis and histologic type were analyzed. A written form of informed consent was obtained from every patient, and the study was approved by the Clinical Research Ethics Committee of Shanghai Jiao Tong University

2.2. Quantitative real-time PCR (q-PCR)

Total RNA samples were extracted from lung cancer tissues or cells with TRIzol regent (TaKaRa). cDNA was synthesised from 1 μg of total RNA with One Step RT-PCR Kit (TaKaRa). q-PCR was performed with the SYBR Green (TaKaRa) detection method on an ABI-7500 RT-PCR system (Applied Biosystems). The primers used in this study is listed in Supplementary Table 1.

2.3. Western blot

Lung cancer tissues and cells were lysed with cell lysis buffer (Beyotime). Total proteins were applied to SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membranes, followed by blocking in the buffer containing 5% fat-free dry milk. The membranes were then probed with indicated antibodies overnight, and then washed and incubated with HRP-conjugated secondary antibodies (Zhongsanjinqiao) for 1.5 h and finally visualized using Chemiluminescent ECL reagent (Vigorous Biotechnology). The following antibodies were used: Anti-GAPDH (Cell Signaling Technology), anti-PHF8 (Cell Signaling Technology), anti-PARP (Abcam), anti-caspase 3 (Abcam), anti-Bax (Abcam), anti-Bcl-2 (Santa Cruz), anti-p21 (Santa Cruz).

2.4. Cell lines and cell culture

A549 and LC-AI were obtained from Riken BioResource Center. NCI-H292 were purchased from ATCC. A549 and LC-AI cells were cultured in high glucose-containing DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. NCI-H292 cells were cultivated in RPMI 1640 with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. All cells were cultured at 37 °C under a humidified atmosphere containing 5% CO_2.

2.5. Retrovirus package, transduction and transfection

Control shRNA and specific sh-RNAs targeting PHF8 were purchased from Invitrogen, and the corresponding sequences were cloned into the pSIREN-RetroQ plasmid (Addgene) for retrovirus production with 293T cells. The targeting sequences were listed in Supplementary Table 2. For transduction, 293T cells were incubated with virus-containing supernatant in the presence of 8 mg/ml polybrene. After 48 h, infected cells were selected for with puromycin (2 mg/ml). Then the clones were picked and cultured for further experiment.

For transfection, the locked nucleic acid (LNA)-antimiR-21 or LNA-control (Exiqon A/S, Skelstedet, Vedbaek, Denmark) were delivered at a final concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen).

2.6. Cell proliferation assay

Cell proliferation was monitored by a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cell proliferation/Viability Assay kit (R&D SYSTEMS) in according to the guidelines.

2.7. Tumor xenograft experiment

Xenograft mice experiments were performed as described previously [16]. In brief, equal numbers of lung cancer cells expressing either control or PHF8 knockdown vectors were injected subcutaneously, within 30 min of harvesting, over the right and left flanks in male nu/nu mice between 4 and 6 weeks of age. The mice were killed and the tumors were weighted 3 weeks after inoculation. $N = 10$ in each group. This experiment was repeated for three times.

2.8. Soft sugar colony formation assay

Lung cancer cells were suspended in 1.5 ml complete medium supplemented with 0.45% low melting point agarose (Invitrogen). The cells were placed in 35 mm tissue culture plates containing 1.5 ml complete medium and agarose (0.75%) on the bottom layer.

Table 1
Clinical characteristics of the patients with NSCLC.

Variables		Total	PHF8 low expression (n = 29)	PHF8 high expression (n = 31)	*P* value
Age	⩾60	32	15	17	1.0000
	<60	28	14	14	
Gender	Male	39	18	21	0.7876
	Female	21	11	10	
Smoking history	Never	22	15	7	**0.0313**
	Ever	38	14	24	
Histology type	Adenocarcinomas	37	17	20	0.7912
	Squamous cell carcinomas	23	12	11	
Tumor size	⩾3 cm	27	6	21	**0.0003**
	<3 cm	33	23	10	
Metastasis and recurrence	Present	19	5	14	**0.0273**
	Absent	41	24	17	
Stage	I	18	12	6	**0.0440**
	II	23	12	11	
	III	19	5	14	

Bold values are those $p < 0.05$.

The plates were incubated at 37 °C with 5% CO_2 for 2 weeks. Cell colonies were stained with 0.005% crystal violet and analyzed using a microscope.

2.9. FACS analysis and TUNEL assay

Cells were washed twice with cold PBS and then re-suspended in 1 × binding buffer (BD Biosciences) at a concentration of 1×10^6 cells/ml. Then, 100 ml of the solution (1×10^5 cells) was transferred to a 5 ml culture tube and stained with 5 ml each of allophycocyanin–annexin V (BD Biosciences) and 50 mg/ml propidium iodide (Invitrogen). The cells were gently mixed and incubated at room temperature for 15 min. For assessment of apoptosis, 400 ml of 1 × binding buffer was then added to each tube and the samples were analyzed by flow cytometry using a LSRII instrument (Becton–Dickinson).

The induction of apoptosis was also monitored by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. The TUNEL assay was performed in according to the guidelines recommended of the TUNEL Apoptosis Kit (R&D SYSTEMS).

2.10. Statistical analysis

All values are expressed as the mean ± SEM of at least three independent experiments. Statistical differences between two groups were determined using Student's *t* test. The correlation of PHF8 mRNA level with patients' clinicopathological variables was analyzed by the $\chi 2$ test or Fisher's exact test. The correlation of PHF8 level and miR-21 level was analyzed with linear regression analysis. The Kaplan–Meier method was used to estimate overall survival. Survival differences according to PHF8 expression were analyzed by the log-rank test. Statistical analyses were performed using GraphPad Prism. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. PHF8 high expression predicts poor survival

To explore whether PHF8 participates in human lung cancer, we first tested the mRNA and protein levels of PHF8 in human NSCLC tissues and adjacent normal lung tissues. The results indicated that PHF8 was overexpressed in human lung cancer tissues compared to normal lung tissues at both mRNA level and protein level (Fig. 1A and B), and PHF8 level was correlated with disease stage (Fig. 1B; Table 1). We next analyzed the correlation between PHF8 mRNA level and survival using Kaplan–Meier method. Significantly, high PHF8 level predicted poor overall and disease-free survival (Fig. 1C and D). In addition, high expression of PHF8 was correlated with large tumor size, remote metastasis and high disease stage (Table 1). These findings strongly indicates that PHF8 is involved in human NSCLC.

3.2. PHF8 regulates lung cancer cell growth and transformation

To further investigate the potential role of PHF8 in human lung cancer, we designed two sh-RNAs targeting PHF8 (Supplementary Table 2). When infected with retrovirus carrying sh-PHF8, PHF8 protein level was significantly decreased in A549, LC-AI, and NCI-H292 lung cancer cells (Supplementary Fig. 1A–C). Then we infected lung cancer cells with retrovirus carrying sh-PHF8 and evaluated the effect of PHF8 on lung cancer cell growth. We found that cell proliferation was markedly reduced when PHF8 was knocked down in A549, LC-AI, and NCI-H292 cells (Fig. 2A–C; Supplementary Fig. 2A). We next explored whether PHF8 could regulate lung cancer cell growth *in vivo*, thus we performed tumor xenograft experiment. Significantly, PHF8 knockdown could also attenuate lung cancer cell growth *in vivo* (Fig. 2D–F; Supplementary Fig. 2B). We next probed the potential contribution of PHF8 to the transformative properties of lung cancer cells. We observed that PHF8-depleted cells possessed reduced colony-forming activity in A549, LC-AI, and NCI-H292 cells (Fig. 2G–I;

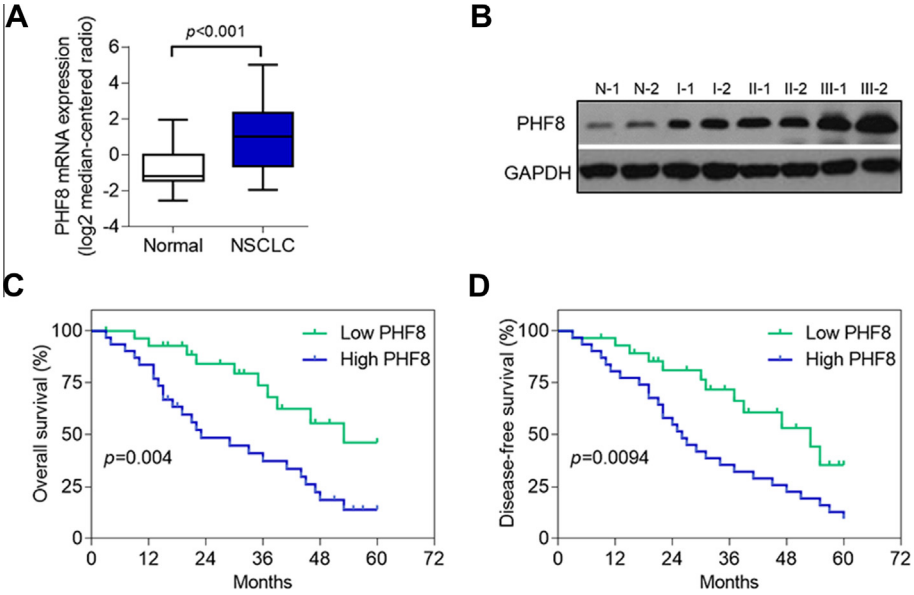

Fig. 1. (A) High PHF8 level predicts poor survival. PHF8 mRNA level is up-regulated in human lung cancer. The mRNA level of PHF8 in adjacent normal tissues and cancer tissues of NSCLC patients was analyzed with q-PCR. $N = 17$ in normal group, $n = 60$ in NSCLC group. (B) Representative western blot showing PHF8 protein level is up-regulated in human lung cancer. Protein was extracted from adjacent normal lung tissues and lung cancer tissues from different stages, and the protein was subjected to Western blot analysis with indicated antibodies. *N*: normal lung tissues; I: Stage I; II: Stage II; III: Stage III. (C and D) Kaplan–Meier curve comparing time to survival between NSCLCs with the low (<25th percentile) vs. high (>25th percentile) PHF8 expression was determined using the q-PCR results. (C) Overall survival. (D) Disease-free survival.

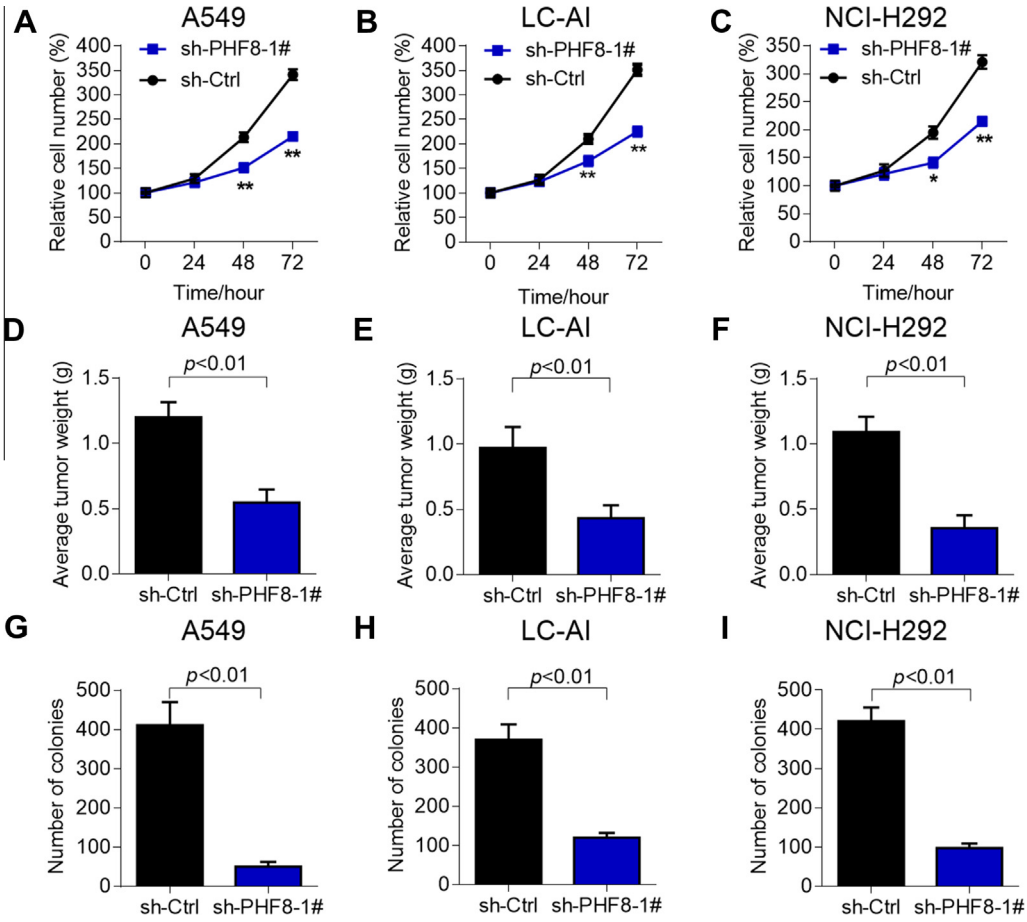

Fig. 2. PHF8 knockdown reduces lung cancer growth and transformation. (A–C) PHF8 knockdown attenuates human lung cancer cell proliferation. A549 (A), LC-AI (B) and NCI-H292 (C) cells were infected with retrovirus carrying sh-Ctrl, sh-PHF8-1# and the cell numbers were evaluated with MTT method at 24, 48, and 72 h post infection, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. sh-Ctrl. (D and E) PHF8 knockdown represses *in vivo* growth of human lung cancer cells. Control lung cancer cells or those with sh-PHF8-1# transduction were subjected to xenograft mice experiments. Tumor weight was evaluated at the terminal of the experiments. $N = 10$ in each group. (D) A549 cells, (E) LC-AI cells, (F) NCI-H292 cells. (G–I) PHF8 knockdown inhibits lung cancer cell transformation. Control cells or those with sh-PHF8-1# transduction were subjected to soft sugar colony formation assay. The number of colonies were analyzed two weeks later. (G) A549 cells, (H) LC-AI cells, (I) NCI-H292 cells.

Supplementary Fig. 2C). In summary, these evidence demonstrate that PHF8 promotes lung cancer cell growth and cellular transformation.

3.3. PHF8 knockdown induces apoptosis in lung cancer cells

To investigate whether PHF8 regulates lung cancer cell proliferation and colony formation by orchestrating apoptosis, we performed FACS to detect apoptotic cells. We found that PHF8 knockdown significantly increased the number of apoptotic cells in A549, LC-AI, and NCI-H292 cells (Fig. 3A and B). We further performed western blot to analyze the apoptotic signaling pathways. The results indicated that PHF8 knockdown activated apoptotic pathway and inactivated antiapoptotic pathway. In detail, PHF8 knockdown increased the protein level of Bax, p21, cleaved caspase 3, and cleaved PARP, while the antiapoptotic protein Bcl-2 was down-regulated when PHF8 was knocked down (Fig. 3C). We next performed TUNEL assay to investigate whether DNA damage is involved in apoptosis induced by PHF8 knockdown. Markedly, PHF8 knockdown increased number of TUNEL-positive cells in lung cancer cells, indicating that PHF8 knockdown induced DNA damage (Fig. 3D). Taken together, PHF8 regulates lung cancer cell survival.

3.4. PHF8 regulates miR-21 in lung cancer

miR-21 is widely accepted as an oncogenic microRNA and its role has been deeply investigated in human NSCLC [17]. miR-21 was reported to be an important anti-apoptotic factor and regulates drug resistance in human NSCLC [18–21]. We found that PHF8 knockdown significantly reduced the level of miR-21 in A549 lung cancer cells (Fig. 4A). Our q-PCR results also showed that miR-21 was up-regulated in human NSCLC tissues (Fig. 4B), which was consistent with previous reports [19,22]. Furthermore, we performed linear regression analysis to figure out whether miR-21 level is correlated with PHF8 expression in human NSCLC. The results indicated that miR-21 level was significantly and positively correlated with PHF8 level in human NSCLC tissues (Fig. 4C). Taken together, these results demonstrate that PHF8 regulates miR-21 expression in human lung cancer.

3.5. miR-21 knockdown blocks the effect of PHF8 on lung cancer cells

As we have demonstrated that PHF8 regressed the expression of miR-21, we wanted to know whether miR-21 is essential for the function of PHF8 in human lung cancer. We knocked down miR-21 in A549 cells using specific LNA-antimiR-21 (Fig. 4D). We found that miR-21 knockdown reduced A549 cell proliferation and

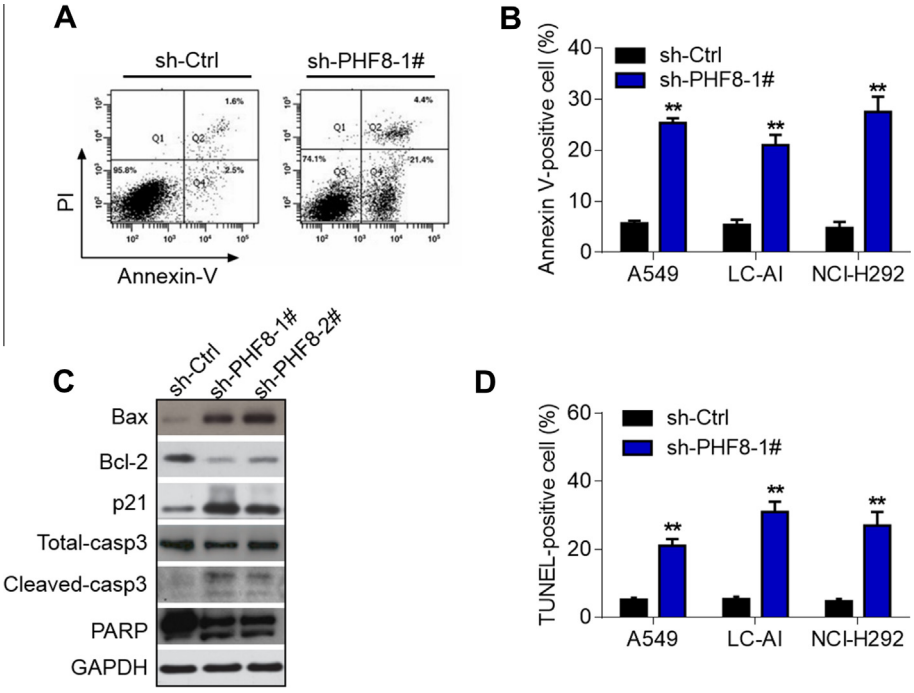

Fig. 3. PHF8 knockdown induces lung cancer cell apoptosis. (A and B) Lung cancer cells were infected with retrovirus carrying sh-Ctrl of sh-PHF8 for 48 h. Percentage of apoptotic cells was analyzed with FACS. (A) Representative FACS analysis of annexin V and propidium iodide (PI) staining of A549 cells infected with retrovirus carrying sh-Ctrl or sh-PHF8. (B) PHF8 knockdown promotes apoptosis (Annexin V-positive) of A549, LC-AI and NCI-H292 cells. $^{**}p < 0.01$ vs. sh-Ctrl. (C) Representative Western blot showing PHF8 knockdown activates caspase 3, Bax, p21, PARP and inhibits Bcl-2 in A549 cells. A549 cells were infected with retrovirus carrying sh-ctrl or sh-PHF8 for 48 h, then western blot was performed to analyze protein level. (E) PHF8 knockdown increases percentage of TUNEL-positive cells. Lung cancer cells were infected with retrovirus carrying sh-Ctrl or sh-PHF8 for 48 h, then TUNEL assay was performed to analyzed DNA damage.

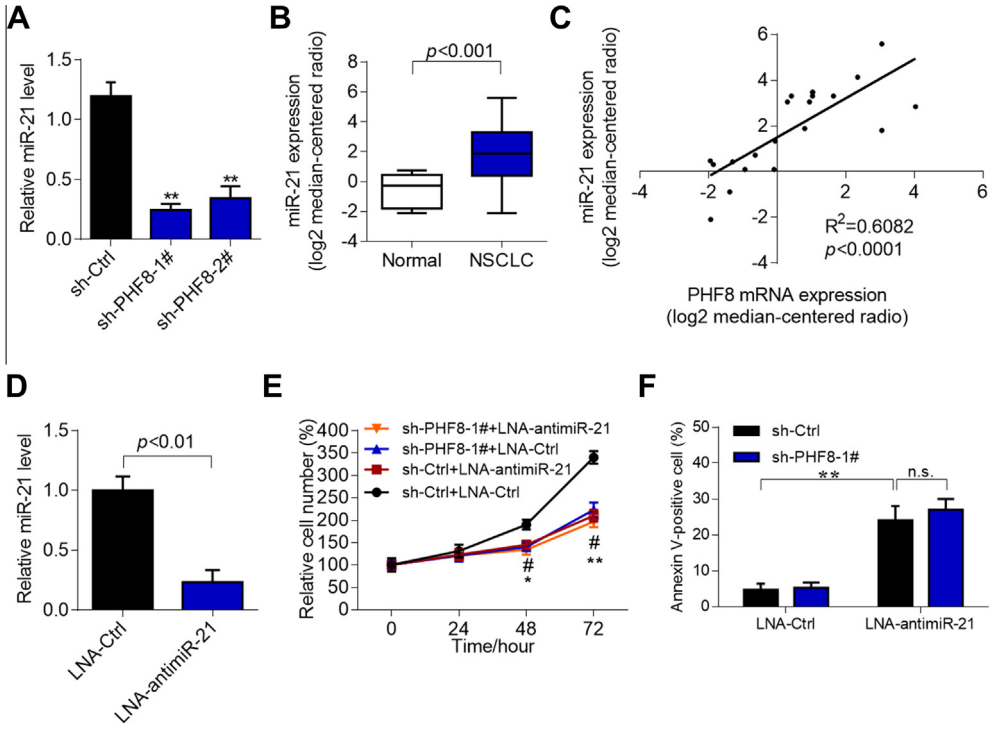

Fig. 4. PHF8 regulates miR-21 in lung cancer cells. (A) PHF8 knockdown decreases the expression of miR-21 in A549 lung cancer cells. A549 cells were infected with retrovirus carrying sh-PHF8 for 48 h. The expression of miR-21 was evaluated with q-PCR. (B) miR-21 is overexpressed in human NSCLC. The level of miR-21 in adjacent normal tissues and cancer tissues of NSCLC patients was analyzed with q-PCR. $N = 10$ in normal group, $n = 21$ in NSCLC group. (C) miR-21 expression is correlated with the level of PHF8. Linear regression analysis was performed to analyze the correlation between miR-21 level and PHF8 level $N = 21$. (D) miR-21 knockdown in A549 cells. A549 cells were transfected with LNA-ctrl or LNA-antimiR-21 for 48 h, then RNA was harvested and subjected to cDNA synthesis and q-PCR. (E) miR-21 knockdown blocks PHF8 effect on A549 cell proliferation. A549 cells were transfected with LNA-ctrl or LNA-antimiR-21 for 24 h and then were infected with indicated retrovirus and cell number was evaluated with MTT method at 24, 48, and 72 h post infection. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ indicate sh-Ctrl+LNA-Ctrl vs. sh-Ctrl + LNA-antimiR-21; $^{**}p < 0.05$, $^{**}p < 0.01$ indicate sh-Ctrl+LNA-Ctrl vs. sh-PHF8-1#+LNA-Ctrl. (F) miR-21 knockdown blocks PHF8 effect on A549 cell apoptosis. A549 cells were transfected with LNA-ctrl or LNA-antimiR-21 for 24 h and then were infected with indicated retrovirus and percentage of apoptotic cells was analyzed with FACS. $^{**}p < 0.01$; n.s.: no significance.

induced apoptosis (Fig. 4E and F). However, PHF8 knockdown was unable to affect A549 cell proliferation and apoptosis when miR-21 was knockdown (Fig. 4E and F). These results demonstrate that miR-21 is critically essential for the function of PHF8 in human lung cancer.

4. Discussion

Here, we define an oncogenic role of PHF8 in human NSCLC. We discovered that the histone demethylase PHF8 is overexpressed in human NSCLC tissues and high expression of PHF8 predicts poor survival (Fig. 1 and Table 1). Interestingly, we found that smoking history is associated with high expression of PHF8 (Table 1), indicating that PHF8 overexpression may be induced by nicotine, which is a cancerogen for human lung cancer. By using *in vitro* and *in vivo* evidence, we demonstrate that PHF8 facilitates human lung cancer cell growth and cellular transformation (Fig. 2), confirming the oncogenic role of PHF8 in human NSCLC.

PHF8 has diverse roles in cellular activity. PHF8 is recruited to promoters by its PHD domain based on interaction with H3K4me2/3 and controls G1-S transition in conjunction with E2F1, HCF-1 and SET1A by removing the repressive H4K20me1 mark from a subset E2F1-regulated gene promoters [3]. In addition, PHF8 is regulated by APC (cdc20) and plays an important role in the G2/M transition [23]. In prostate cancer cells, PHF8 regulates cell migration, motion paths and invasive properties as well as cell proliferation [13]. Knockdown of PHF8 in ESCC cells resulted in inhibition of cell proliferation and an increase of apoptosis. Moreover, there were reductions of both anchorage-dependent and –independent colony formation [15]. *In vitro* migration and invasion assays showed that knockdown of PHF8 led to a reduction in the number of migratory and invasive cells. Furthermore, downregulation of PHF8 attenuated the tumorigenicity of ESCC cells *in vivo* [15]. In the present study, we found that PHF8 knockdown inhibits cellular proliferation of lung cancer cells (A549, LC-AI, NCI-H292) *in vitro* and *in vivo*. Furthermore, cellular transformation of lung cancer cells was also reduced by PHF8 knockdown (Fig. 2). In addition, we showed that PHF8 knockdown induces apoptosis in lung cancer cells by up-regulating apoptotic protein (Bax, p-21, cleaved caspase 3 and cleaved PARP) and down-regulating the antiapoptotic protein Bcl-2. Noticeably, DNA damage is involved in this effect (Fig. 3). Together with previous reports, our evidence indicate that PHF8 regulates cellular proliferation and transformation behaviors of cancer cells.

MiR-21 is one of the most prominent miRNAs implicated in the genesis and progression of human cancer. Not only has it been implicated in the promotion of tumor growth [17], proliferation [24], antiapoptosis [25] and response to gemcitabine-based chemotherapy [26], but diverse studies have shown that miR-21 is overexpressed in different tumor types. MiR-21 is overexpressed in human lung cancer and serve as a diagnostic and prognostic factor [20–22,27,28], which is consistent with our results (Fig. 4B). MiR-21 overexpression promotes growth, metastasis and chemoresistance in NSCLC cells by targeting PTEN [19,28]. Importantly, miR-21 is an antiapoptotic factor in lung cancer cells [18]. However, how miR-21 is regulated in human lung cancer cells remains unknown. Here, we identify PHF8 as an upstream regulator of miR-21. PHF8 promotes miR-21expression in human lung cancer cells, and in turn, miR-21 mediates the function of PHF8 in human lung cancer cells because miR-21 knockdown blocks PHF8 effects on lung cancer growth and apoptosis (Fig. 4).

In conclusion, PHF8 is an oncogenic protein in human NSCLC and could serve as a prognostic factor. PHF8 regulates lung cancer cell proliferation, cellular transformation and survival, at least in part, through maintaining the expression of miR-21. PHF8 may serve as a promising prognostic marker and a candidate target for intervention.

Conflict of interest

None.

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.2014.07.076.

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