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Histone demethylase PHF8 promotes cell growth and metastasis of non-small-cell lung cancer through activating Wnt/β-catenin signaling pathway

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Summary. PHD finger protein 8 (PHF8), serving as a histone demethylase, is upregulated in some types of malignant tumors. The role of PHF8 in non-small-cancer lung carcinoma (NSCLC) remains unclear. This study aims to verify the effect of PHF8 in NSCLC and its molecular mechanism. We collected 20 cases of fresh NSCLC and adjacent lung tissues to assess differential expressions of PHF8 by reverse transcription-quantitative PCR (RT-qPCR). Western blot was employed to examine protein levels of PHF8, Wnt1, β-catenin and epithelial-mesenchymal transition (EMT) related proteins. Chromatin immunoprecipitation assays were executed to confirm the regulatory mechanism of PHF8 and Wnt1. Cell Counting Kit-8 assays and Transwell assays were utilized to identify the effects of PHF8/Wnt1 pathway on cell proliferation, migration and invasion. PHF8 was overexpressed in NSCLC tissues and cells and higher PHF8 expression was correlated with poorer overall survival in NSCLC patients. PHF8 overexpression promoted NSCLC cell proliferation, migration and invasion, while PHF8 knockdown exerted the opposite effect. Mechanistic investigations identified that PHF8 occupied the Wnt1 promoter, leading to a decrease of repressive histone markers H3K9me1, H3K9me2, H3K27me2 and H4K20me1 in the promoter region of the Wnt1 gene, which further promoted the transcription of the Wnt1 gene. PHF8 activated Wnt/β-catenin signaling pathway through promoting Wnt1 expression. Besides, PHF8 altered the EMT of NSCLC through regulating Wnt1 levels. PHF8, acting as an oncogene and prognostic biomarker in NSCLC, stimulated NSCLC to proliferate, metastasis and EMT by activating Wnt/β-catenin signaling.

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Introduction

Over 80% of lung cancers are pathologically identified as non-small-cell lung cancer (NSCLC), accounting for more than one million human mortalities worldwide each year (Siegel et al., 2020). Lung adenocarcinoma (LUAD) and lung squamous carcinoma (LUSC) are the most general subtype of NSCLC. In spite of the new developments in early diagnosis, the adjuvant and neoadjuvant therapies, the average survival for aggressive NSCLC patients is only 8-10 months (Bray et al., 2018; Siegel et al., 2020). Therefore, it is very important to elucidate the mechanisms involved in the pathogenesis of NSCLC and develop new personalized molecular-targeted therapies.

Histone methylation, controlled by methyl-transferase and demethylase, is a widespread pattern of epigenetic regulation. Histone methylation was reported to play a vital role in the occurrence and metastasis of different tumors (Michalak et al., 2019; Thakur and Chen, 2019). PHF8 (PHD finger protein 8), a histone demethylase, is responsible for catalyzing the removal of methyl groups from monomethylated and dimethylated H3 lysine 9 (H3K9me1/2), monomethylated histone H4 lysine 20 (H4K20me1) and dimethylated H3 lysine 27 (H3K27me2), serving as a transcription coactivator (Kleine-Kohlbrecher et al., 2010; Liu et al., 2010; Qi et al., 2010). For instance, PHF8 was confirmed to transcriptionally upregulate FOXA2 by demethylating and removing the repressive histone markers on the promoter region of the FOXA2 gene in neuroendocrine prostate cancer (Liu et al., 2021). PHF8 functioned as a transcriptional coactivator of HER2 gene and promoted resistance to the anti-HER2 effects of breast cancer cells (Liu et al., 2020). Collectively, these findings indicate that PHF8 acts as a transcriptional co-activator and

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participates in cellular processes.

PHF8 has been involved in playing oncogenic roles in various types of malignancies, including esophageal squamous cell carcinoma (Sun et al., 2013), neuroendocrine prostate cancer (Liu et al., 2021), acute promyelocytic leukemia (Arteaga et al., 2013), gastric cancer (Li et al., 2017) and breast carcinogenesis (Wang et al., 2016). PHF8 participates in a variety of biological processes, including apoptosis, cell cycle, proliferation, metastasis, epithelial-mesenchymal transition (EMT), and DNA damage protection (Bjorkman et al., 2012; Tong et al., 2016; Wang et al., 2016; Li et al., 2017; Shao et al., 2017; Feng et al., 2020). Moreover, PHF8 was also reported to be an oncogenic protein and could serve as a prognostic factor in human NSCLC (Shen et al., 2014). However, the function and regulatory mechanism of PHF8 in NSCLC still need more investigation.

Here, we found that PHF8 acted as an oncogene and prognostic biomarker in NSCLC and PHF8 promoted lung cancer cell proliferation, migration and invasion. Furthermore, we confirmed that PHF8 functioned as a transcriptional coactivator of the Wnt1 gene and activated the Wnt/β-catenin signaling pathway. Our study may provide a potential therapeutic target for NSCLC diagnosis and treatment.

Materials and methods

Human tissue sample

A total of 20 fresh primary NSCLC tissues and matched adjacent noncancerous lung tissues were obtained from patients at The First People's Hospital of Zigong City. None of these patients had received radiotherapy or chemotherapy prior to surgery. The study protocol was approved by the Ethics Committee of The First People's Hospital of Zigong City (Approve no.2020013). All patients diagnosed with NSCLC had been confirmed based on pathological assays. Informed consent was written and provided by all patients. Tissue was flash-frozen in liquid nitrogen before long-term storage at -80°C.

Cell culture

Human NSCLS cell lines, A549, PC9, H1299, H1650 and human normal lung epithelial cell line HBE were purchased from ATCC. All cells were cultured in RPMI-1640 medium (Hyclone, Logan, USA) supplemented with 10% FBS (Gibco, Grand Island, USA) at 37°C and 5% CO_2.

Cell transfections

Small interfering RNAs (siRNAs) (Guangzhou RiboBio Co, Ltd, Guangzhou, China) were used to transiently downregulate PHF8 expression in cells with scrambled siRNA as the controls. The specific siRNAs against PHF8 were as follows: si-PHF8-1, 3'-CCGGAGACAGTGCGAACCGTA-5'; si-PHF8-2, 3'-TCGGCGAACCAAGATAGCAAA-5'. For PHF8 and Wnt1 overexpression, the full length of PHF8 and Wnt1 mRNA was cloned into pcDNA3.1 vector. Lipofectamine 2000 (Invitrogen, Carlsbad, USA) was applied to transfect plasmids and siRNAs into cells according to the manufacturer's instruction. 48 h after transfection, the cells were harvested for further analysis.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, USA). Total RNA was reverse transcribed into cDNA using a SuperScript Reverse Transcriptase kit (Thermo Fisher Scientific, Waltham, USA). Then, cDNA was detected with the HiScript® II One Step qRT-PCR SYBR® Green Kit (Takara, Tokyo, Japan). GAPDH was used as the internal control for normalization. The fold changes were calculated by means of relative quantification ($2^{-\Delta\Delta Cq}$ method). The primers were listed as follows: PHF8 sense, 3'-GCAAACCGCAGCACCACACCT-5' and anti-sense, 3'-CGAGTCTCTGCTTTGCTGTG-5'; Wnt1 sense, 3'-CTCATGAACCTTCACAACAACGA-5' and anti-sense, 3'-ATCCCGTGGCACTTGCA-5'; GAPDH sense, 3'-TGTGGGCATCAATGGATTTGG-5' and anti-sense, 3'-ACACCATGTATTCCGGGTCAAT-5'.

Western blot

NSCLS cells were lysed with cell lysis buffer (Beyotime, Shanghai, China). Total proteins were separated by SDS-polyacrylamide gel and electrophoretically transferred to polyvinylidene fluoride membranes. After blocking with 5% nonfat milk, the membranes were then probed with the indicated antibodies overnight. After washing, the membranes were subsequently incubated with HRP-conjugated secondary antibodies (Abcam, Cambridge, USA) for 1 hour. Chemiluminescent ECL reagent (Vigorous Biotechnology, Beijing, China) was used to visualize. The following primary antibodies were used: anti-GAPDH (Cell Signaling Technology, Beverly, USA), anti-PHF8 (Cell Signaling Technology), anti-Wnt1 (Abcam), anti-E-cadherin (Abcam), anti-N-cadherin (Abcam), anti-vimentin (Abcam) and anti-GAPDH (Abcam).

Transwell assays

24 well Transwell chambers (Corning, NY, USA) without pre-coated Matrigel were used to assess cell migration ability and Transwell chambers pre-coated with 50 ng/μl Matrigel solution (BD Biosciences, San Jose, USA) were used for cell invasion assay. Transfected H1299 and H1650 were seeded into the top chamber at a density of 8×10^4 cells per well in 200 μl of

RPMI-1640 medium. The bottom chambers were filled with 800 µl of RPMI-1640 medium with 10% FBS. After incubation for 48 h, cells were mechanically removed from the upper membrane surface. Migrated or invaded cells on the lower surface of membrane were counted under a microscope after the cells were fixed in 4% paraformaldehyde for 20 min and dyed with 0.1% crystal violet staining solution (Beyotime, Nantong, China) for 10 min.

Cell proliferation assay

1×10^4 transfected cells were seeded onto 96-well plates per well for 0, 24, 48 and 72 h. 10 µl of Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Jiangsu, China) was added to each well. Then, absorbance was determined at 450 nm, after all cells were incubated at 37°C for 2h.

Chromatin immunoprecipitation assays

ChIP assay used Simple ChIP Enzymatic Chromatin IP Kit (Cell signaling Technology, Danvers, USA). The transfected H1299 cells grown in 10-cm plates were cross-linked with 1% formaldehyde and quenched with 0.125M glycine. After washing with cold PBS, cells were lysed. DNA fragments ranging from 200 to 500 bp were generated via sonication. Then the lysates were immunoprecipitated with control IgG (A01008, GenScript, USA), anti-PHF8 (ab36068, Abcam), anti-H3K27me2 (ab24684, Abcam), anti-H3K9me1 (ab8896, Abcam), anti-H3K9me2 (ab1220, Abcam) and anti-H4K20me1 (ab9051, Abcam). Immunoprecipitated DNAs were analyzed by qRT-PCR.

Statistical analysis

GraphPad Prism software (v6.02) was employed to conduct statistical analysis. Results are displayed as the means ± SD. Differences between experimental groups were compared using an unpaired two-tailed Student's t-test (for two conditions) or one way of ANOVA (for more than three conditions). Kaplan-Meier survival curve was analyzed with log-rank test. A P value <0.05

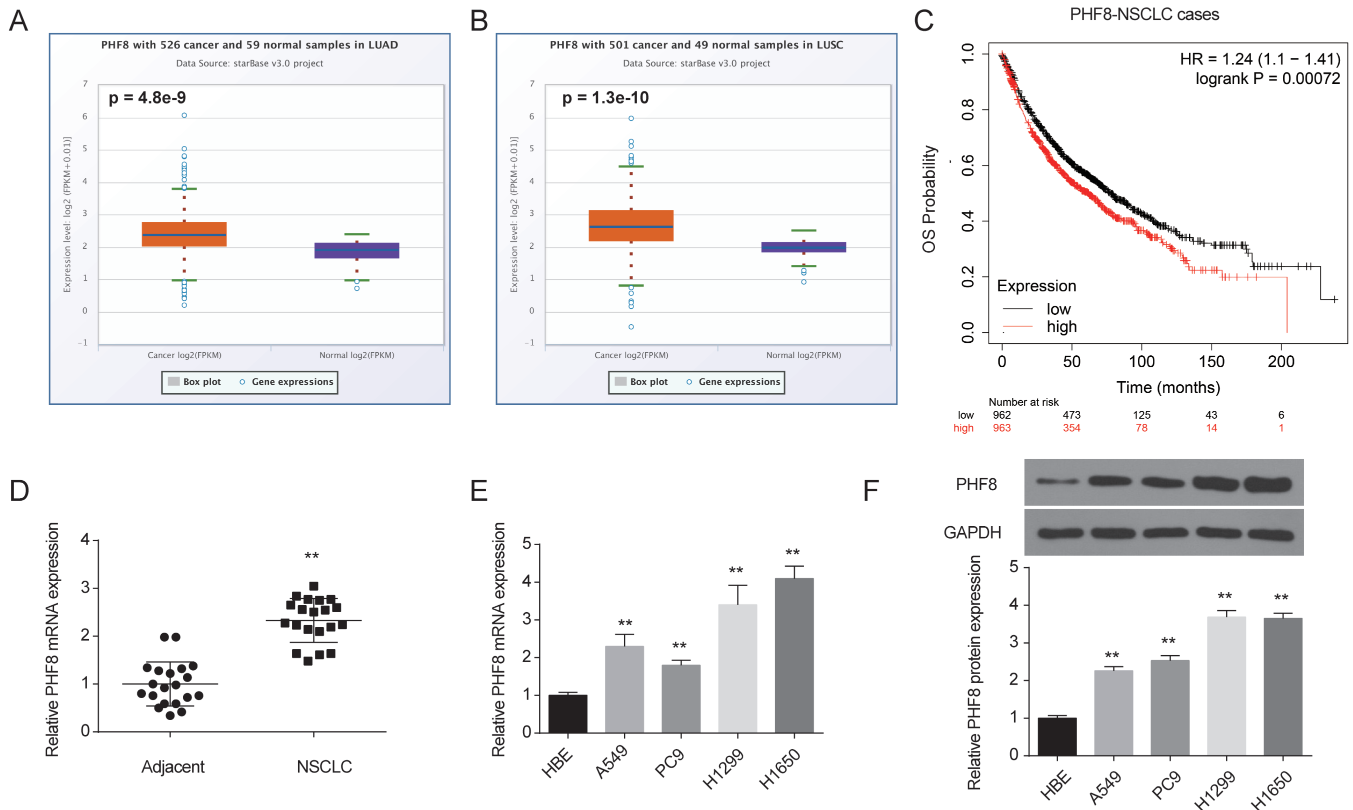

Fig. 1. PHF8 was upregulated in NSCLC tissues and cells. **A.** PHF8 levels in LUAD were presented and the data were from LUAD dataset in ENCORI database. **B.** PHF8 levels in LUSC were presented and the data were from LUSC dataset in ENCORI database. **C.** The overall survival (OS) was analyzed and compared between patients with low and high levels of PHF8 in NSCLC patients from database of Kaplan-Meier plotter. **D.** The expression of PHF8 in tumor tissues was significantly higher than that in normal adjacent tissues as shown by qRT-PCR. ** P<0.01 compared with adjacent group. **E.** The expression of PHF8 in NSCLC cell lines (H1299, PC9, H1299, and H1650) was markedly higher than that in normal HBE cells as shown by qRT-PCR. ** P<0.01 compared with HBE group. **F.** PHF8 protein levels in NSCLC cell lines were upregulated as detected by western blot. ** P<0.01 compared with HBE group.

was considered statistically significant.

Results

PHF8 is overexpressed in NSCLC

Based on the database of ENCORI (The Encyclopedia of RNA Interactomes), PHF8 was found to be up regulated in LUAD and LUSC tissues (Fig. 1A-B). To further explore the clinical significance of PHF8 in NSCLC, the public database of Kaplan-Meier plotter analysis (http://www.kmplot.com) was employed. Higher PHF8 expression was correlated with poorer overall survival (OS) in NSCLC (Fig. 1C). To further verify whether PHF8 expression was indeed upregulated, we detected PHF8 mRNA levels in 20 NSCLC tissues and paired adjacent normal tissues and found that PHF8 levels were dramatically higher in NSCLC tissues than those in the adjacent tissues (Fig. 1D). What's more, PHF8 mRNA and protein levels were also confirmed to be upregulated in 4 human NSCLC cell lines when compared with the normal bronchial epithelial cell line (HBE) (Fig. 1E-F). The expression levels of PHF8 mRNA were higher in H1299 and H1650 cells when compared with A549 cells, while there was no significant change between A549 or PC9 cells. PHF8 mRNA levels were higher in H1299 and H1650 cells compared with PC9 cells. There was no significant change in PHF8 mRNA expression between H1299 and H1650 cells. Similar to the mRNA levels, PHF8 proteins expression were found to be enhanced in H1299 and H1650 cells compared with A549 or PC9 cells. There was no significant change in PHF8 protein expression between A549 or PC9 cells, as well as between H1299 and H1650 cells. The proteins expression of PHF8 were in concert with mRNA expression of PHF8 in NSCLC cell lines. In short, PHF8 was upregulated in NSCLC tissues and cells and might be implicated in the malignant process of NSCLC.

PHF8 controls NSCLC cell growth and metastasis

Given that PHF8 is overexpressed in NSCLC tissues and cells, we speculated that it may act as an oncogene in NSCLC. To test this hypothesis, we firstly upregulated PHF8 expression by transfecting exogenously expressed PHF8 into H1299 and H1650 cells (Fig. 2A). We downregulated the expression levels of PHF8 in H1299 and H1650 cells by two different siRNA constructs (Fig. 2B). Next, the CCK8 assay was used to detect the role of PHF8 in cell viability. Ectopic expression of PHF8 significantly promoted cell growth in both cell lines (Fig. 2C-D). PHF8 knockdown visibly inhibited cell growth in H1299 and H1650 cells (Fig. 2E-F). Apart from proliferation, metastasis is also a vital aspect of cancer progression. As shown in Fig. 3A-B, the migration and invasion abilities of H1299 and H1650 were enhanced after ectopic expression of PHF8. PHF8 knockdown significantly decreased the migrating and invading cell numbers in H1299 and H1650 cells (Fig.

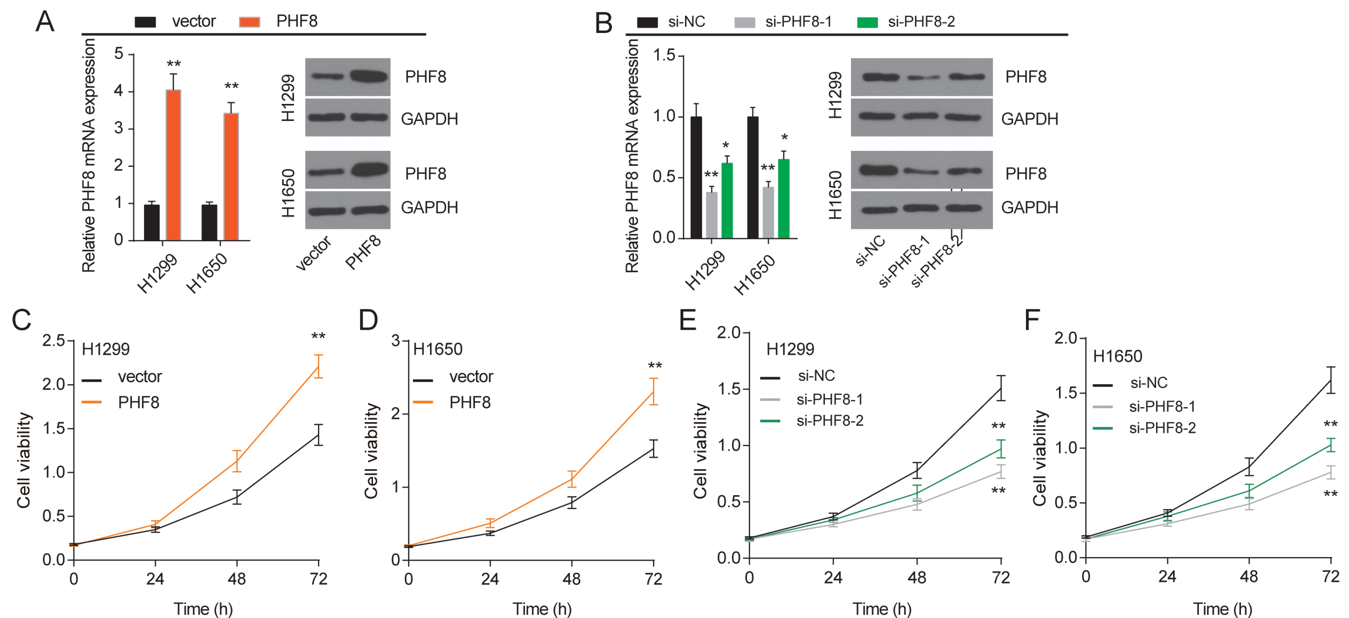

Fig. 2. PHF8 overexpression promoted H1299 and H1650 cells growth. **A.** The relative expression of PHF8 in H1299 and H1650 cells was detected by qRT-PCR and western blot after PHF8 overexpression. * P<0.05, ** P<0.01 compared with vector group. **B.** The relative expression of PHF8 in H1299 and H1650 cells was detected by qRT-PCR and western blot after being treated with siRNA for PHF8. ** P<0.01 compared with si-NC group. **C, D.** CCK8 was used to detect the cell viability of H1299 and H1650 cells after PHF8 overexpression. ** P<0.01 compared with vector group. **E, F.** CCK8 was used to detect the cell viability of H1299 and H1650 cells after PHF8 knockdown. ** P<0.01 compared with si-NC group.

3C-D). In short, those results illustrated that PHF8 functions as an oncogenic driver in NSCLC.

PHF8 actives Wnt/β-catenin signaling pathway through enhancing Wnt1 transcription

The Wnt/β-catenin pathway is a highly conserved signaling pathway that controls diverse physiological and pathological processes including carcinogenesis. As presented in Fig. 4A, PHF8 and Wnt1 levels in NSCLC tissues showed a positive correlation. We next analyzed ChIP-Seq data of A549 downloaded from the Encyclopedia of DNA Elements (ENCODE) database and found that PHF8 was highly enriched in the Wnt1 promoter regions (Fig. 4B). PHF8 was reported to catalyze the removal of methyl group from H3K9me1/2, H3K27me2 and H4K20me1 (Qi et al., 2010). We further explored whether PHF8 was involved in transcriptional regulation in a demethylase activity-dependent manner. The chromatin immunoprecipitation assay showed that PHF8 overexpression enhanced PHF8 occupancy of the Wnt1 promoter, with a concurrent decrease of repressive histone markers H3K9me1/2, H3K27me2 and H4K20me1 in the promoter region of the Wnt1 gene. PHF8 knockdown decreased PHF8 occupancy of the Wnt1 promoter, with a concurrent increase of repressive histone markers H3K9me1/2, H3K27me2 and H4K20me1 in the promoter region of the Wnt1 gene (Fig. 4C-G). Besides, we demonstrated that PHF8 overexpression increased mRNA levels of Wnt1 while PHF8 knockdown decreased mRNA levels of Wnt1 in H1299 and H1650 cells (Fig. 4H). Finally, we found that PHF8 overexpression enhanced the protein levels PHF8, Wnt1 and β-catenin while PHF8 knockdown decreased the protein levels PHF8, Wnt1 and β-catenin in H1299 and H1650 cells (Fig. 4I). Collectively, our results supported the notion that PHF8 actived the wnt/β-catenin signaling pathway through enhancing Wnt1 transcription.

PHF8 affects NSCLC cell growth, metastasis and EMT through the Wnt1/β-catenin signaling pathway

To investigate whether Wnt1 is indeed required for the observed phenotypes caused by PHF8 silencing in H1299 and H1650 cells, we transfected exogenously expressed Wnt1 into PHF8 knockdown cells. Wnt1 overexpression reversed the inhibition effect of Wnt1/β-catenin signaling induced by PHF8 knockdown in H1299 and H1650 cells (Fig. 5A). As shown in Fig. 5B-

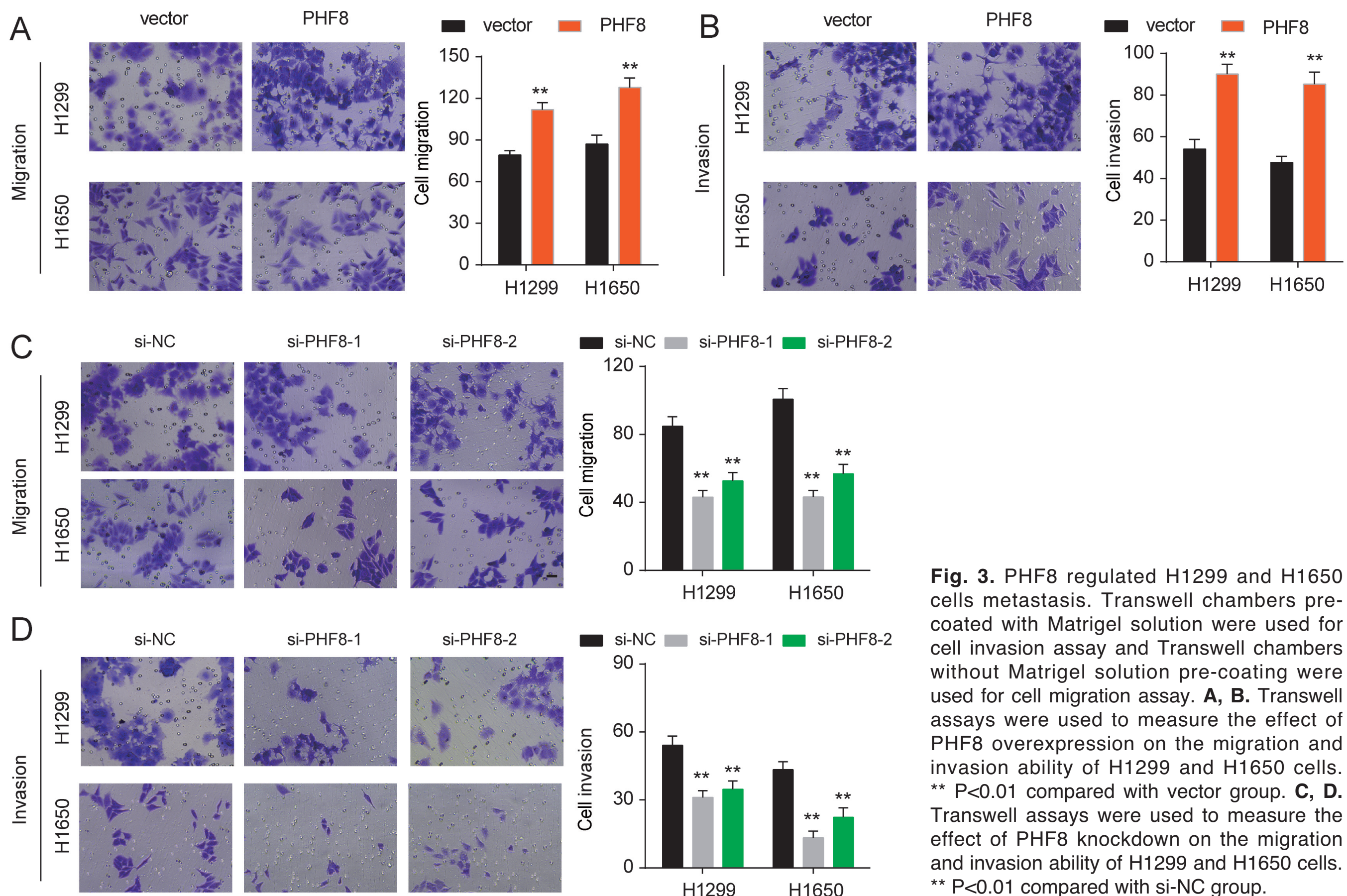

Fig. 3. PHF8 regulated H1299 and H1650 cells metastasis. Transwell chambers pre-coated with Matrigel solution were used for cell invasion assay and Transwell chambers without Matrigel solution pre-coating were used for cell migration assay. **A, B.** Transwell assays were used to measure the effect of PHF8 overexpression on the migration and invasion ability of H1299 and H1650 cells. ** P<0.01 compared with vector group. **C, D.** Transwell assays were used to measure the effect of PHF8 knockdown on the migration and invasion ability of H1299 and H1650 cells. ** P<0.01 compared with si-NC group.

C, the defects in cell growth induced by PHF8 knockdown were partially rescued by overexpression of Wnt1. Similarly, restoration of Wnt1 expression partially reversed the decreased migrating and invading cell numbers in H1299 and H1650 cells induced by PHF8 silencing (Fig. 5D-E). EMT is a process in which epithelial cells acquire mesenchymal features and is associated with tumor initiation, invasion, metastasis,

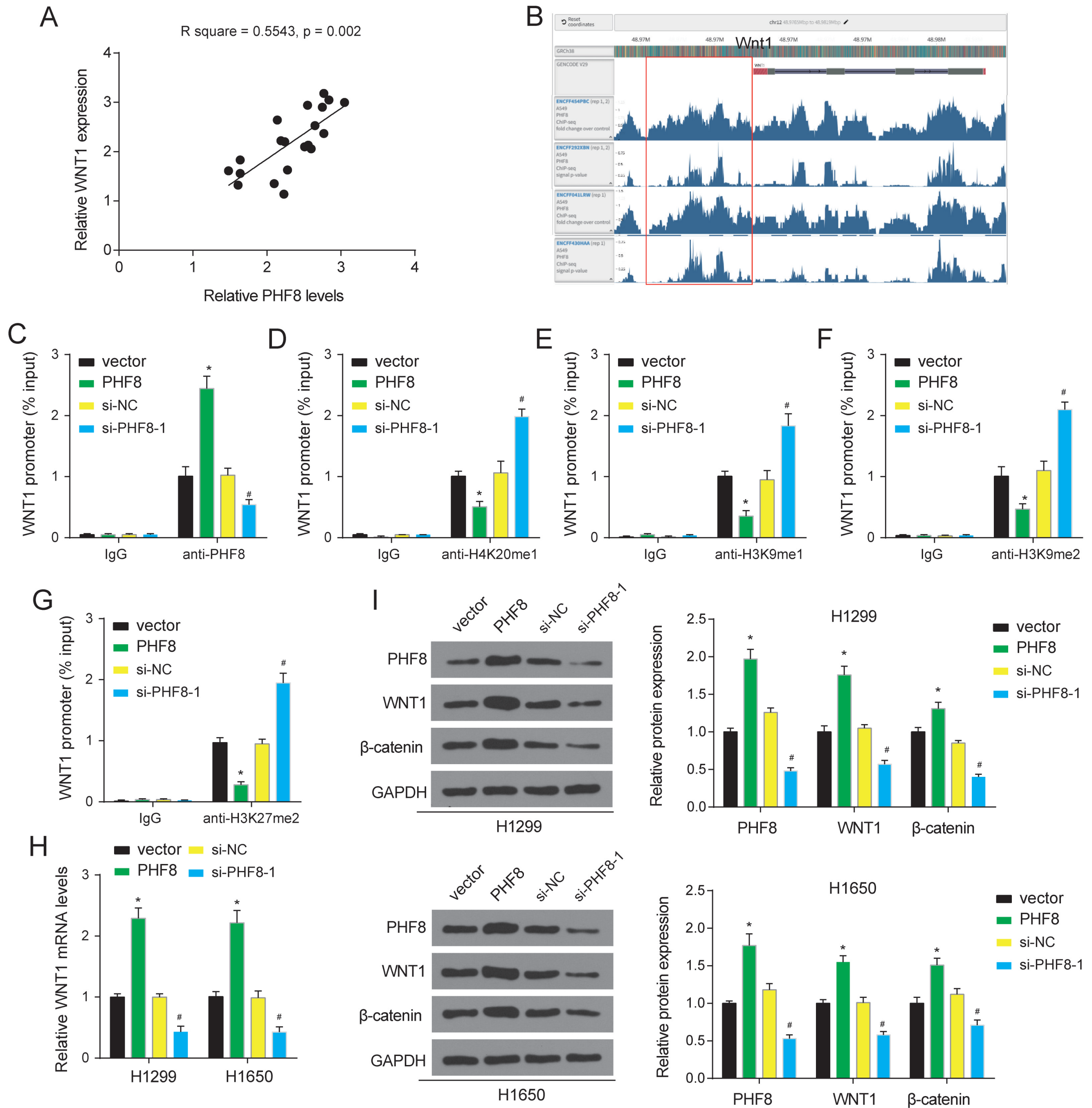

Fig. 4. PHF8 actived Wnt/β-catenin signaling pathway through enhancing Wnt1 transcription. **A.** PHF8 and Wnt1 levels in NSCLC tissues showed a positive correlation. **B.** Analysis of PHF8 ChIP-seq of A549 cells in the Wnt1 locus. **C-G.** H1299 cells with indicated transfection were subjected to ChIP assays with PHF8, H4K20me1, H3K9me1, H3K9me2 and H3K27me2 antibodies. The immunoprecipitated materials were used for qRT-PCR analyses of the promoter regions of Wnt1. **H.** Wnt1 mRNA levels were measured by qRT-PCR after PHF8 overexpression or knockdown. **I.** PHF8, Wnt1 and β-catenin protein levels were measured by western blot after PHF8 overexpression or knockdown. * P<0.05 compared with vector group, # P<0.05 compared with si-NC group.

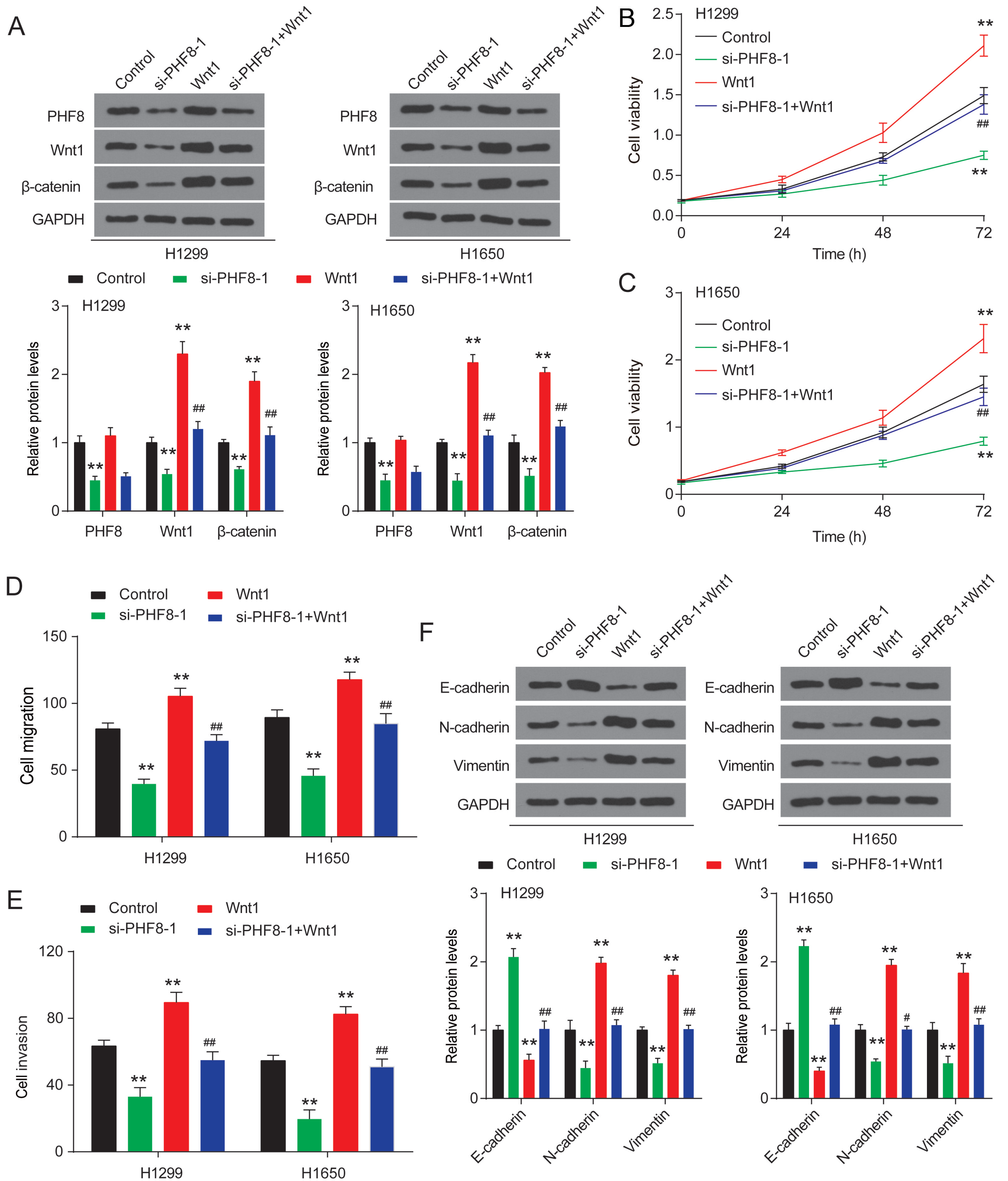

Fig. 5. Wnt1 overexpression restored the inhibition effect of PHF8 knockdown on cell growth, metastasis and EMT. **A.** Wnt signaling related protein levels were measured after PHF8 knockdown or Wnt1 overexpression in H1299 and H1650 cells. **B, C.** CCK8 was used to detect the cell viability in H1299 and H1650 cells after PHF8 knockdown or Wnt1 overexpression. **D, E.** Transwell assays were used to measure the migration and invasion ability of H1299 and H1650 cells after PHF8 knockdown or Wnt1 overexpression. **F.** Epithelial-mesenchymal transition related proteins were detected in H1299 and H1650 cells after PHF8 knockdown or Wnt1 overexpression. ** P<0.01 compared with control group; # P<0.05, ## P<0.01 compared with si-PHF8-1 group.

and resistance to therapy. In our study, we also found that PHF8 knockdown upregulated E-cadherin protein levels, and downregulated N-cadherin and Vimentin protein levels, which indicated that PHF8 knockdown inhibited EMT of NSCLS. Wnt1 overexpression reversed the inhibition effect on EMT induced by PHF8 knockdown (Fig. 5F). Therefore, these results revealed that PHF8 exerted its biological functions on cell growth, migration, invasion, and EMT via regulating Wnt1 in NSCLC.

Discussion

Here, we have identified an oncogenic role of PHF8 in human NSCLC. PHF8 was highly expressed in NSCLC tissues and cells. High expression of PHF8 predicts poor survival in NSCLC patients. Similar to our results, Shen et al. also reported the oncogenic function in NSCLC and they found that PHF8 regulated NSCLC cell growth, transformation and apoptosis by promoting miR-21 expression (Shen et al., 2014). In our study, we also confirmed the role of PHF8 on NSCLC cell growth and found that PHF8 promoted both H1299 and H1650 cell proliferation. Furthermore, we also verified the promotion effect of PHF8 on NSCLC cell migration, invasion and EMT. H1299 and H1650 cells were specially chosen to further study, which was different from Shen's report in which A549 and LC-AI cells were selected (Shen et al., 2014), indicating the general function of PHF8 on NSCLC.

PHF8 targets various genes in human cancers and has the functions of pro-tumor and pro-metastasis (Bjorkman et al., 2012; Tong et al., 2016; Li et al., 2017; Shao et al., 2017). Similarly, our results suggested that PHF8 positively regulated tumor proliferation, migration, invasion and EMT of NSCLC cells. Notably, PHF8 is reported to transcriptionally activate EMT-related protein expression, including SNAI1 and VIM, and reduce E-cadherin levels (Li et al., 2017; Shao et al., 2017). PHF8 upregulation promotes autophagic degradation of E-cadherin and further promotes EMT and metastasis of hepatocellular carcinoma (Zhou et al., 2018). In our study, we found that PHF8 controls the expression of EMT-related markers, E-cadherin, N-cadherin and vimentin through co-transcriptionally activating Wnt1 expression. Our data further supplemented the regulatory mechanism of PHF8 and indicated that PHF8 could be a potential target for gene therapy of NSCLC.

Wnt/β-catenin signaling is vital for maintenance of cell homeostasis as well as mammalian development (Moon et al., 2004). It is widely known that the Wnt/β-catenin signaling pathway activated in many human cancers regulates cell proliferation, migration and invasion (Moon et al., 2004; Clevers, 2006). The abnormal expressions of Wnt/β-catenin signaling pathway related genes are involved in NSCLC invasion and metastasis (Kahlert et al., 2013). In our study, we found that PHF8 and Wnt1 levels in NSCLC tissues showed a positive correlation. PHF8 was able to occupy the Wnt1 promoter, leading to a decrease of repressive histone markers H3K9me1/2, H3K27me2 and H4K20me1 in the promoter region of the Wnt1 gene, which further promoted the transcription of Wnt1 gene. We firstly reported that Wnt1 was a direct downstream target of PHF8. In this study, we discovered that PHF8 activated the Wnt/β-catenin signaling pathway through promoting Wnt1 expression. PHF8 is also reported to physically interact with β-catenin, leading to the promotion of mesenchymal markers transcription in gastric cancer (Li et al., 2017). Given that in our study β-catenin was found to be upregulated after PHF8 overexpression, this mechanism could potentially be at play also in NSCLC.

Metastasis and malignant proliferation are the common hallmarks of tumors (Hanahan and Weinberg, 2011). PHF8, as an epigenetic regulator, was reported to activate transcription of various metastasis and proliferation-related genes. For example, silencing PHF8 in hepatocellular carcinoma cells significantly decreased the cells' ability for proliferation, migration, invasion and sphere formation through regulating CUL4A (Ye et al., 2019). PHF8 knockdown inhibits proliferation and promotes the apoptosis of adult acute lymphoblastic leukemia cells *in vitro* as well as attenuating tumor growth *in vivo* via transcriptionally upregulating MEK1 (Fu et al., 2018). Here, we identified Wnt1 as a target gene of PHF8 and PHF8 might perform its tumor promotion function via the Wnt/β-catenin signaling pathway. PHF8 might promote cell growth, migration and invasion and the inhibition induced by PHF8 knockdown was partially rescued by the overexpression of Wnt1.

The major limitation of this study was the lack of *in vivo* study. While these cell experiments might closely measure the characteristics of *in vivo* study, the accuracy would be improved if an *in vivo* study was conducted. Moreover, clinical practices were not fully investigated to testify the outcome of this study.

Conclusions

In summary, our study demonstrates that PHF8 acts as an oncogenic regulator partially relying on its histone demethylase activity. PHF8 is overexpressed in NSCLC and its overexpression is related to the proliferative and metastatic phenotype, predicting poor prognosis in NSCLC patients. We revealed a core role of the epigenetic regulator PHF8 in NSCLC development, indicating PHF8 function as a potential therapeutic target for the deadly NSCLC.

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Data Availability. The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Approval. This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics

Committee of the First People's Hospital of Zigong City.

Consent Informed. Consent was obtained from all individual participants included in the study.

Competing interests. The authors declare that they have no conflicts of interest.

Authors' Contributions. All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Yan Hu, and Ying Yang. The first draft of the manuscript was written by Hanshuo Mu. Revising it critically for important intellectual content and final approval of the version to be submitted were done by Yan Hu and Hanshuo Mu. All authors read and approved the final manuscript.

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